THE JOURNAL OF NUTRITION

VOLUME 33

JANUARY–JUNE, 1947

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA, PA.

CONTENTS

No. 1 JANUARY, 1947

No. 2 FEBRUARY, 1947

No. 3 MARCH, 1947

No. 4 APRIL, 1947

No. 5 MAY, 1947

No. 6 JUNE, 1947

DEFICIENCIES OF CERTAIN VITAMINS AS STUDIED WITH TURKEY POULTS ON A PURIFIED DIET

I. PTEROYLGLUTAMIC ACID, RIBOFLAVIN, NIACIN AND INOSITOL

T. H. JUKES, E. L. R. STOKSTAD AND MARGARET BELT
Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York

SIX FIGURES

(Received for publication September 26, 1946)

Difficulties have been encountered with turkeys, as with chicks, when attempts have been made to devise purified diets which will permit normal growth. Now that synthetic pteroylglutamic acid is available (Angier et al., '46) it has been found possible by means of its inclusion to prepare diets in which the water-soluble vitamins are supplied in synthetic form and which give satisfactory growth in turkeys.

EXPERIMENTAL

Day-old turkey poults were placed in electrically heated battery brooders and were fed the experimental diets immediately. Ten or eleven Bronze or White Holland turkeys were used in each group.

In preliminary experiments glucose was used as the source of carbohydrate. Difficulties occurred, including stickiness and caking of glucose on the feathers, the occurrence of "pendulous crops" as early as 3 weeks of age, and high mortality. Since the first of these difficulties was thought to be due to the physical properties of glucose, a change was made to corn

starch, and no further difficulties were encountered. The possibility remained that the starch may have contained some unidentified ingredient which was responsible in part for the improvement.

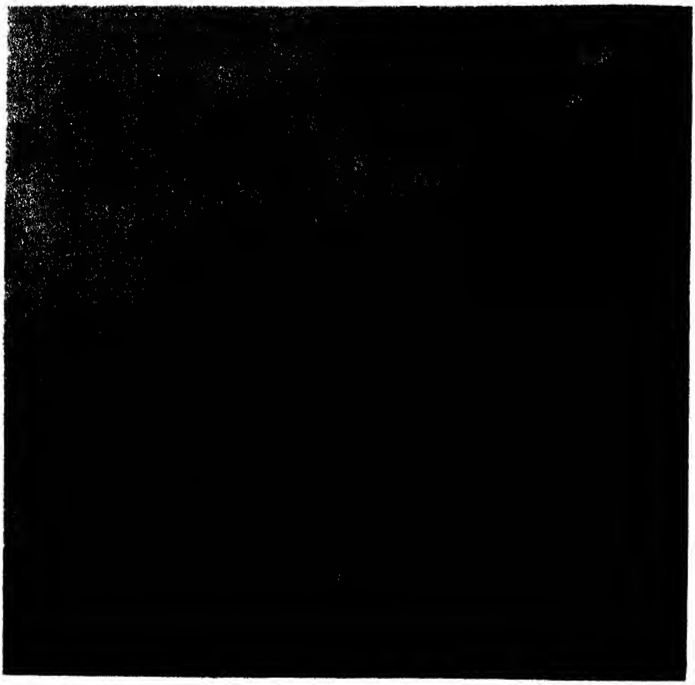


Fig. 1 Turkey on diet T-15 at 11 weeks of age, weight 2348 gm, and of comparatively normal appearance except for a slight enlargement of the crop.

The following basal diet was used: starch 55.5 gm, purified casein [1] 20 gm, gelatin 8 gm, calcium gluconate 5 gm, glucose [2] 0.88 gm, cystine 0.4 gm, choline chloride 0.2 gm, inositol 0.1 gm, bone ash 2 gm, NaCl 0.6 gm, KH_2PO_4 0.45 gm, K_2HPO_4 0.6 gm, $MgSO_4$ 0.25 gm, $MnSO_4$, 4 H_2O 0.05 gm, ferric citrate 0.05 gm, $CuSO_4$, 5 H_2O 2 mg, $Al_2(SO_4)_3$, 18 H_2O 1.6 mg, KI 0.6 mg, cobalt chloride 0.4 mg, nickel chloride 0.2 mg, calcium

[1] Labco.

[2] Cerelose.

pantothenate 5 mg, niacinamide 5 mg, riboflavin 1 mg, pyridoxine HCl 1 mg, thiamine HCl 1 mg, p-aminobenzoic acid 1 mg, 1-acetoxy-2 methyl-4 naphthyl sodium phosphate 0.5 mg, sodium pteroylglutamate 2 mg, (dl) biotin .04 mg, to which were added vitamin A 3000 U.S.P. units, vitamin D 400 A.O.A.C. units, mixed tocopherols 68 mg, dissolved in corn oil[3] to a total of 6 gm.

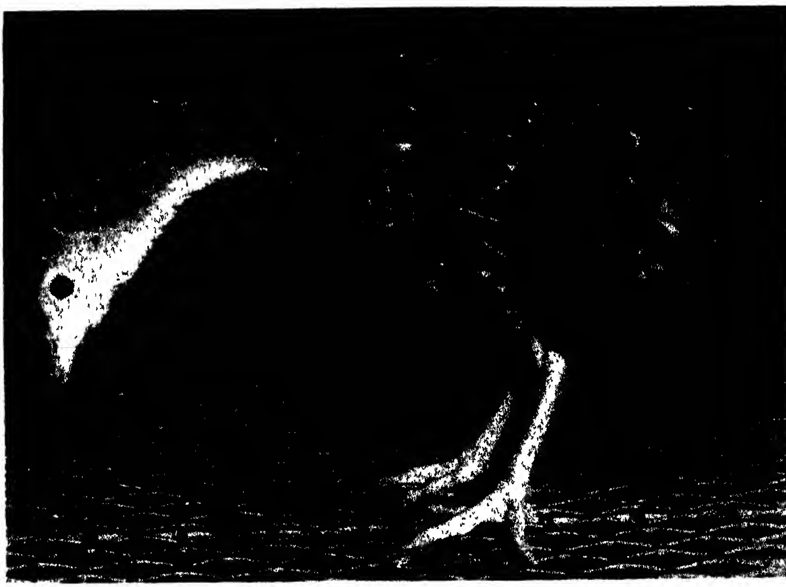


Fig. 2 Cervical paralysis in turkey in group 1, deficient in pteroylglutamic acid. Age 33 days.

The water-soluble vitamins were added as follows: inositol, niacinamide, calcium pantothenate, thiamine, riboflavin, pyridoxine, p-aminobenzoic acid and 1-acetoxy-2 methyl-4 naphthyl sodium phosphate were mixed dry with glucose so that 1 gm of the mixture was sufficient for 100 gm of diet. (dl) biotin was dissolved, 0.8 mg per ml, in 50% alcohol with the aid of a drop of ammonium hydroxide solution and the biotin solution was dried on the glucose before the other vitamins were mixed with it. Sodium pteroylglutamate was prepared by

[3] Mazola.

dissolving pteroylglutamic acid in just sufficient sodium hydroxide solution and making up to a final concentration of 2 mg of pteroylglutamic acid per ml in 50% ethanol. The solution was added to the diet. Turkeys on this diet ("diet T-15") appeared normal (fig. 1), and grew at a fair rate, although not at the maximum rate which might be anticipated. Blood was drawn from a wing vein for hemoglobin determinations. The blood was hemolyzed with dilute ammonium hydroxide and readings were made in the Coleman spectrophotometer at 540 mμ. The calculations were made from a standard curve which was constructed on the basis of a comparison of the readings obtained for a series of hemoglobin solutions with both the Evelyn colorimeter and the Coleman spectrophotometer. Cell volume determinations were made by centrifuging blood in standard Wintrobe hematocrit tubes. Smears were stained supravitally with brilliant cresyl blue and counterstained with Wright's solution.

Pteroylglutamic acid

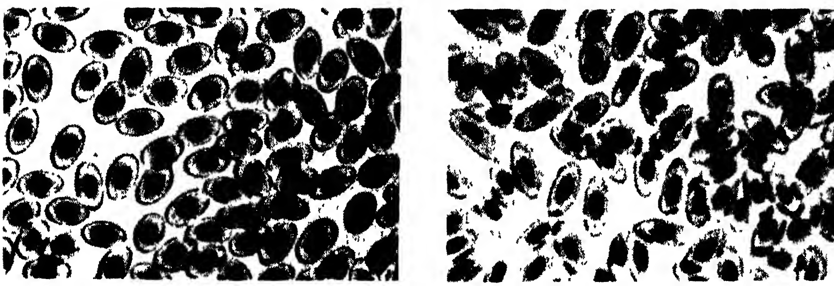
A deficiency of this vitamin resulted in slow growth and a moderate degree of anemia. The results are shown in table 1.

TABLE 1

Effect of various levels of pteroylglutamic acid on turkey poults fed a basal purified diet consisting of diet T-15 with pteroylglutamic acid omitted.

GROUP	SUPPLEMENT PER KILO OF DIET	WEIGHT IN GM AT			PER CENT HEMOGLOBIN AT		AT 4 WEEKS	
		21 days	28 days	42 days	3 weeks	4 weeks	RBC count	Mean corpuscular volume
1	None	165	191	all dead	8.65	10.42	2.43	1.12
2	0.2 mg pteroylglutamic acid	201	284	409	9.19	10.66	2.50	1.04
3	0.5 mg pteroylglutamic acid	194	287	565	9.46	10.04	2.71	0.99
4	0.8 mg pteroylglutamic acid	225	324	646	9.66	10.05	2.65	1.04
5	1.0 mg pteroylglutamic acid	200	315	607	9.35	10.23	2.68	1.06

Three cases of cervical paralysis (Richardson, Hogan and Kempster, '45) were observed among 4 surviving birds on the basal diet at 33 to 35 days of age (fig. 2). The results indicate that growth was somewhat better at a level of 0.8 mg of pteroylglutamic acid per kilo of diet than at a level of 0.5 mg. Increasing the level to 1.0 mg did not increase the growth rate. Examination of blood smears revealed a difference in appearance between the erythrocytes from turkeys



3A 3B

Fig. 3 A Normal-appearing erythrocytes (× 1500) from turkey in group 5, receiving a diet supplemented with 1.0 mg pteroylglutamic acid per kilo. B. Erythrocytes (× 1500) from turkey in group 1, deficient in pteroylglutamic acid. Note the large size and elongation of the cells as compared with the normal erythrocytes. Some of the cells show nuclei which are enlarged and of diminished density. The blood smears were made at 28 days of age.

in group 1 with pteroylglutamic acid deficiency and the erythrocytes from turkeys in group 5. The erythrocytes of the deficient birds were larger in diameter and their nuclei were larger and appeared less dense than in the case of the birds receiving supplementary pteroylglutamic acid (fig. 3).

Riboflavin

Omission of riboflavin from the basal diet resulted in slow growth and dermatitis as summarized in table 2. The dermatitis (fig. 4) was observed in the region of the eyes and mouth and corresponded closely to the original descriptions of riboflavin-deficiency dermatitis in turkeys (Lepkovsky and Jukes,

'36; Jukes, '38). The requirement for riboflavin under the conditions of the experiment appeared to be satisfied by 3.0 mg per kilo of diet. A level of 2.5 mg per kilo was insufficient for growth and did not give complete protection against dermatitis.

An additional group was included in the series. The group (group 9 A) received the same diet as group 9, but with casein [4] replacing gelatin in the diet. The object was to obtain

TABLE 2

Effect of various levels of riboflavin on turkey poults when added to a basal purified diet consisting of diet T-15 with riboflavin omitted.

EXP.	GROUP	SUPPLEMENT PER KILO OF DIET	WEIGHT IN GM AT			PER CENT OF BIRDS SHOWING DERMATITIS AT		
			21 days	28 days	42 days	17 days	25 days	42 days
1	6	None	134	170	210	56		80
1	7	2.5 mg riboflavin	199	254	457	25		0
1	8	3.0 mg riboflavin	228	340	610	0		0
1	9	4.0 mg riboflavin	226	338	640	0		0
1	9A	Same diet as group 9 but with casein replacing gelatin	187	260	404	0		0
2	19	None	105 [1]	117 [2]	150†	75	66 [2]	
2	20	2.5 mg riboflavin	139	152	198	11	44	57
2	21	3.0 mg riboflavin	120	150	210	11	33	25
2	22	4.0 mg riboflavin	168	244	429	0	22	11
2	23	10.0 mg riboflavin [3]	189	266	472	0	0	0

[1] 5 survivors † 1 survivor.

[2] 3 survivors.

[3] 1.2 mg pteroylglutamic acid per kilo of diet.

some indication as to whether turkeys, like chickens (Almquist and Mecchi, '40), require glycine in the diet for rapid growth. The results indicated that such was the case.

A second experiment with riboflavin was carried out with a group of turkeys which were hatched in August. The results of this experiment are also summarized in table 2 and indicate a higher riboflavin requirement than was observed in the first experiment with this vitamin. The turkeys in the

[4] Labco.

second experiment did not grow as rapidly on higher levels of riboflavin as did the turkeys in the first experiment and dermatitis appeared in groups 21 and 22 which received, respectively, 3.0 mg and 4.0 mg of riboflavin per kilo of diet. The slow growth which was obtained in experiment 2, and which was observed even in the case of group 23, may have been associated with the commonly observed fact that turkeys hatched late in the breeding season tend to grow more slowly and to exhibit less vitality than turkeys which are hatched at the height of the breeding season.

TABLE 3

Effects of various levels of niacinamide on turkey poults when added to a basal purified diet consisting of diet T-15 with niacinamide omitted.

GROUP	SUPPLEMENT PER KILO OF DIET	WEIGHT IN GM AT			PER CENT INCIDENCE OF PEROSIS AT		
		21 days	28 days	42 days	18 days	27 days	42 days
10	None	92 [1]	all dead		0 [1]	all dead	
11	20 mg niacinamide	158	215	341	33	89	100
12	50 mg niacinamide	182	273	479	0	0	0
13	100 mg niacinamide	174	263	487	0	0	0

[1] One survivor.

Niacin

Without the addition of niacin to the diet, only 1 of 10 birds survived to the age of 18 days as indicated in table 3, group 10. Perosis (fig. 5) occurred in birds in group 11 which received the diet containing 20 mg of added niacinamide per kilo; this confirms the report by Briggs ('46). No abnormalities were observed in the birds in groups 12 and 13 which received 50 or 100 mg of added niacinamide per kilo of diet. Growth was more rapid in groups 12 and 13 than in group 11.

Inositol

The results with inositol are summarized in table 4. Growth was improved by adding 1.0 gm of inositol per kilo but not by 0.1 gm. The hemoglobin level was lowered somewhat by omitting inositol from the diet.

Fig. 4 Dermatitis and rough feathering in turkey in group 6, deficient in riboflavin. Age 21 days.

Fig. 5 Perosis in turkey in group 11, receiving a diet containing a suboptimal level of niacinamide. Age 42 days.

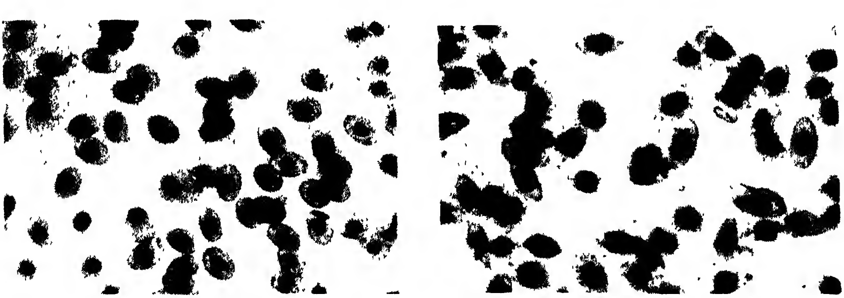
TABLE 4

Effect of various levels of inositol on turkey poults when added to a basal purified diet consisting of diet T-15 with inositol omitted.

EXP.	GROUP	SUPPLEMENT PER KILO OF DIET	WEIGHT IN GM AT 21 days	28 days	40 days	PER CENT HEMOGLOBIN AT 21 days	33 days	42 days
1	14	None	139	194	290	8.28	9.16	
1	15	0.1 gm inositol	141	192	271			
1	16	1.0 gm inositol	166	234	416	9.57	9.98	
2	17	None	137	171	221	9.18	10.32	10.30
2	18	1.0 gm inositol	159	209	322	11.30	10.37	12.24

An examination was made of stained blood smears from anemic turkeys in group 17 (table 4) at 6 weeks of age. The erythrocytes had an appearance which indicated a normocytic anemia, and which was characterized by approximately normal cell diameter, the cells round rather than oval in appearance, the nuclei enlarged and exhibiting irregular color density, and the cytoplasm staining a deeper blue than in normal cells. The erythrocytes in corresponding smears obtained from group 18 were normal in appearance (fig. 6).

The presence of inositol, as phytic acid, in avian erythrocytes was reported by Rapoport ('40). It was later noted



6A 6B

Fig. 6 A. Normal-appearing erythrocytes (× 1500) from turkey in group 18 on diet supplemented with 1 gm of inositol per kilo. B. Erythrocytes (× 1500), showing normocytic anemia, obtained from turkey in group 17, deficient in inositol. Note the paleness of the cytoplasm, the anisocytosis and the enlargement of the nuclei. Some immature red cells, characterized by darker cytoplasm and especially large nuclei, are present. The blood smears were made at 42 days of age.

(Rapoport and Guest, '41) that turkey blood contained 74.2 mg of phytic acid phosphorus per 100 ml of packed cells. Phytic acid was not detected by these investigators in the blood of 22 mammalian species.

DISCUSSION

The observation regarding the paralytic condition associated with pteroylglutamic acid deficiency in turkeys (Richardson et al., '45) has been confirmed in the present investigation. The present report indicates that the pteroylglutamic acid requirement of young turkeys may be at least twice that of chicks. Other investigations (Russell, W. C., '46, private communication) indicate that the pteroylglutamic acid requirement of young turkeys under certain conditions may be even higher than the levels studied in the present investigation.

In a previous investigation (Jukes, '38) it was noted the amount of riboflavin required in the diet of turkey poults was about the same as that required by chicks. The first experiment in the present report tends to support this finding. The second experiment, which was made with "late-hatched" turkeys, indicated a higher riboflavin requirement. The riboflavin requirement of young turkeys was investigated with a diet of natural foodstuffs and was estimated as 2.7 mg per kilo of diet (Patrick et al., '44). In a recent investigation, the riboflavin requirement of young turkeys appeared to be in the neighborhood of 3.5 mg per kilo of diet (Bird et al., '46). Dermatitis was noted which was attributed to biotin deficiency in the basal diet. Perosis was noted on suboptimal levels of riboflavin. These observations may be contrasted with another report (McGinnis and Carver, '46) in which riboflavin was found to be very effective in preventing dermatitis in young turkeys.

The niacin requirement appeared to be satisfied by 50 mg of niacinamide per kilo of ration but 20 mg was insufficient.

Inositol deficiency in turkeys has not previously been described. The deficiency resulted in growth and a normocytic anemia. A level of 1.0 gm of inositol per kilo of diet produced

more rapid growth than was produced by 0.1 gm. Levels higher than 1.0 gm were not fed.

The growth of turkeys was reduced by substituting casein for gelatin in diet T-15. This may possibly indicate that turkeys, like chicks (Almquist and Mecchi, '40), require glycine for growth.

SUMMARY

1. Young turkeys were found to grow and survive on a purified diet containing synthetic vitamin B complex factors.

2. Slow growth, cervical paralysis, a moderate degree of anemia, and high mortality were observed when pteroylglutamic acid was omitted from the diet. The requirement for pteroylglutamic acid under the conditions of the experiment appeared to be in the neighborhood of 0.8 mg per kilo of diet. A characteristic appearance of the erythrocytes in pteroylglutamic acid deficiency was observed and is illustrated.

3. Riboflavin deficiency resulted in slow growth and dermatitis. A level of 2.5 mg of riboflavin per kilo of diet was not sufficient for growth or protection against dermatitis, but 3.0 mg appeared to be sufficient for the first 6 weeks under the conditions encountered in 1 experiment. A higher requirement, apparently somewhat in excess of 4.0 mg of riboflavin per kilo of diet, was indicated in a second experiment which was made with birds hatched at the end of the breeding season.

4. Omission of niacin from the basal diet resulted in slow growth and high mortality. A level of 20 mg of added niacinamide per kilo of diet enabled the birds to survive but growth was slow and the turkeys developed perosis. A level of 50 mg of added niacinamide appeared to be sufficient for fairly rapid growth and for the prevention of perosis.

5. Inositol deficiency was found to produce slow growth and a normocytic anemia.

6. Substitution of casein for gelatin in the basal diet resulted in slow growth. This may indicate that turkeys, like chicks, require glycine in the diet.

ACKNOWLEDGMENT

The photomicrographs in figures 2 and 6 were supplied by the Stamford Research Laboratories of the American Cyanamid Company.

LITERATURE CITED

ALMQUIST, H. J., AND E. MECCHI 1940 Identification of the rice factor. The essential nature of the glycine component. J. Biol. Chem., *135:* 355.

ANGIER, R. B., J. H. BOOTHE, B. L. HUTCHINGS, J. H. MOWAT, J. SEMB, E. L. R. STOKSTAD, Y. SUBBAROW, C. W. WALLER, D. B. COSULICH, M. J. FAHRENBACH, M. E. HULTQUIST, E. KUH, E. H. NORTHEY, D. R. SEEGER, J. P. SICKELS AND J. M. SMITH, JR. 1946 The structure and synthesis of the liver L. casei factor. Science, *103:* 667.

BIRD, F. H., V. S. ASMUNDSON, F. H. KRATZER AND S. LEPKOVSKY 1946 The comparative requirements of chicks and turkey poults for riboflavin. Poul. Sci., *25:* 47.

BRIGGS, G. M. 1946 Nicotinic acid deficiency in turkey poults and the occurrence of perosis. J. Nutrition, *31:* 79.

JUKES, T. H. 1938 The vitamin G requirements of young poults. Poul. Sci., *17:* 227.

LEPKOVSKY, S., AND T. H. JUKES 1936 The response of rats, chicks, and turkey poults to crystalline vitamin G (flavin). J. Nutrition, *12:* 515.

McGINNIS, J., AND J. S. CARVER 1946 The effect of riboflavin and biotin on dermatitis and perosis in turkey poults. Poul. Sci., *25:* 407.

PATRICK, H., M. I. DARROW AND C. L. MORGAN 1944 The role of riboflavin in turkey poult nutrition. Poul. Sci., *23:* 146.

RAPOPORT, S. 1940 Phytic acid in avian erythrocytes. J. Biol. Chem., *135:* 403.

RAPOPORT, S., AND G. M. GUEST 1941 Distribution of acid-soluble phosphorus in the blood cells of various vertebrates. J. Biol. Chem., *138:* 269.

RICHARDSON, L. R., A. G. HOGAN AND H. L. KEMPSTER 1945 Requirement of the turkey poult for vitamin B_c. J. Nutrition, *30:* 151.

PHOSPHORUS IN POULTRY NUTRITION

III. THE RELATIONSHIP BETWEEN THE SOURCE OF VITAMIN D AND THE UTILIZATION OF CEREAL PHOSPHORUS BY THE POULT [1]

E. P. SINGSEN, L. D. MATTERSON AND H. M. SCOTT
Poultry Department, Storrs Agricultural Experiment Station, University of Connecticut, Storrs

ONE FIGURE

(Received for publication September 18, 1946)

The observations of Bird ('44) and Boucher ('44) that vitamin D_3 is essentially two times as effective as vitamin D from cod liver oil in promoting calcification in poults offered a possible explanation for some of the variations which appear in the literature regarding the vitamin D requirements of the turkey poult. That this difference in efficacy might be associated with the mineral content of the diet is suggested by the data of Fritz, Hopper and Moore ('45), and more specifically with the phytin phosphorus by Matterson, Scott and Singsen ('46). According to Singsen and Mitchell ('45) when chicks were forced to rely upon phytin as their principle source of phosphorus, irradiated animal sterols were more effective in promoting calcification than was cod liver oil. The experiments reported here were conducted in an effort to demonstrate that with the poult, the relationship between the source of vitamin D and the utilization of phytin phosphorus is essentially the same regardless of whether calcium magnesium phytate or the natural cereal ingredients are used to supply the phytin.

[1] Supported in part by a grant from the Yantic Grain and Products Company, Norwich, Connecticut.

A preliminary report of this work was made at the Annual Meeting of the Poultry Science Assn. at Saint Louis, July 23, 1946. The abstract of the paper appears in Poul. Sci., *25:* 411, 1946.

Musseh1 and Ackerson ('35) have shown that normal growth and bone ash may be obtained with turkeys when the levels of phosphorus intake vary from 0.63 to 1.47%. Hammond, McClure and Kellogg ('44) have shown that 0.62% phosphorus and 1.02% calcium would support excellent calcification in poults when 80 A.O.A.C. chick units of vitamin D per 100 gm of feed were fed. Unpublished data obtained at the Storrs Agricultural Experiment Station confirm these findings, in that 0.60% available phosphorus supports normal growth and calcification. Evans and Brant ('45) reported that 0.80% phosphorus, a calcium : phosphorus ratio of 2 : 1 and 100 A.O.A.C. chick units of vitamin D per 100 gm of diet supported excellent calcification. The vitamin D was supplied by what the authors call a "natural vitamin D oil." The diets used in the experiments reported here were planned to contain about 0.60% total phosphorus, a level which is close to the minimum requirement of the turkey poult.

The vitamin D requirements of the turkey poult have been investigated by a number of workers, and values may be found in the literature that range from as low as 80 to over 200 A.O.A.C. chick units per 100 gm of diet. Relatively low levels of vitamin D were chosen for use in our experiments in order to bring out as clearly as possible any differences in efficacy which might exist.

The objects of experiment 1 were, first, to investigate the effect of different levels of cereal phosphorus on bone calcification in the poult when the total calcium and phosphorus intakes and the level of vitamin D are held constant, and second, to measure the effect, if any, of the source of vitamin D on the utilization of cereal phosphorus.

The second experiment was designed to measure the effects on bone calcification (bone ash) of increasing unitages of vitamin D from cod liver oil and irradiated animal sterols (irradiated 7-dehydrocholesterol) with two diets which had been shown in experiment 1 to be rachitogenic because too large a proportion of the total phosphorus was contributed by cereal ingredients. It was our hope to be able to make specific

vitamin D recommendations for turkey poults depending upon (1) the source of vitamin D being used and (2) the total amount of and the sources of phosphorus in the diet.

MATERIALS AND METHODS

The basal diet used in these experiments minus the cereal ingredients and the calcium and phosphorus supplements, appears in table 1.

TABLE 1

Composition of the basal diet.

BASAL MIXTURE		VITAMIN SUPPLEMENT	
	%		*Per 100 lb. diet*
Casein	12.00	Calcium pantothenate	635 mg
Gelatin	3.00	Pyridoxine	227 mg
Primary grown yeast	4.00	Riboflavin	227 mg
Bagasse	4.00	Inositol	227 mg
Liver meal	3.00	Para-aminobenzoic acid	227 mg
Wheat germ oil	1.00	Thiamine	95 mg
NaCl	0.50	Nicotinic acid	95 mg
KCl	0.50	Vitamin K	18 mg
$MnSO_4$	(70 gm)	Choline chloride	45.4 gm

The diet was made up to 100% with additions of ground limestone, bone meal and special white corn meal (a de-germed, de-braned product containing only 0.07% phosphorus, of which less than 0.01% is phytin), or cereal mixture no. 1 containing 40 parts of corn, 20 parts of wheat middlings and 10 parts of wheat bran. Seventy-nine per cent of the total phosphorus contributed by cereal mixture no. 1 was present as phytin. When this cereal mixture comprised 67.52% of the total diet, it contributed 0.42% total phosphorus to the diet. The complete plan of the experiment appears in table 2.

Vitamin D was supplied from either a pure cod liver oil or irradiated 7-dehydrocholesterol (hereafter referred to as D_3) diluted in corn oil. The vitamin D supplements were assayed by the A.O.A.C. chick method in this laboratory and also at the Connecticut (New Haven) Agricultural Experiment Station and assigned a potency on the basis of the combined

assay data. Adequate vitamin A was furnished by the oral administration of a pure vitamin A concentrate in corn oil. The poults received the diet ad libitum. After 28 days they were weighed and sacrificed for bone ash determinations. The dry, fat-free tibia were ashed individually. Total calcium, total phosphorus and phytin phosphorus were determined for all diets. Day-old Broad Breasted Bronze turkey poults from the University of Connecticut flock were used in all experiments.

EXPERIMENTAL

In Experiment 1 two series of lots of poults were fed diets containing increasing amounts of cereal phosphorus and decreasing amounts of bone meal. A Ca : P ratio of approximately 2.5 : 1 was maintained with ground limestone. All lots were fed 80 A.O.A.C. chick units of vitamin D per 100 gm of diet, lots 1–6 receiving pure cod liver oil and lots 7–12 vitamin D_3. Thus the diets fed lots 1 and 7, 2 and 8, etc., were the same except for the source of vitamin D. It was planned to keep the total phosphorus level constant at 0.60% except for lots 6 and 12. These two lots received cereal mixture no. 2 containing more wheat by-products and less corn than mixture no. 1, thus increasing the levels of total and cereal phosphorus fed these lots by 0.17%. This was intended to demonstrate the effect, if any, on bone calcification of increasing the total phosphorus level in the diet by means of the cereal ingredients. Twenty-one poults were started in each lot. Mortality was insignificant, only three poults dying in the entire experiment. A summary of the experimental data on rations and results appears in table 2.

It is obvious from the data presented in table 2 that the per cent bone ash decreases steadily and rapidly, as the relative amount of cereal phosphorus increases. The poults of lot 5 were markedly rachitic despite the presence of 80 units of vitamin D per 100 gm of diet, and adequate levels of calcium and total phosphorus. The downward trend was similar for both sources of vitamin D, but the per cent bone ash of the poults receiving the vitamin D_3 declined less sharply and

never reached as low a point as did those receiving cod liver oil. It is interesting to note that increasing the total phosphorus level 0.17% by means of cereal phosphorus had very little effect upon bone ash when the diet contained cod liver oil (lot 6), but a marked effect when it contained vitamin D_3 (lot 12). In general, poults receiving the D_3 exhibited a bone ash value approximately equal to that of poults receiving cod liver oil and 0.10% more non-cereal phosphorus. The relationship between the level of non-cereal phosphorus and bone

TABLE 2

Summary of experimental data on rations, and results in experiment 1.

DIET VARIABLES	LOT NOS.					
	1 and 7	2 and 8	3 and 9	4 and 10	5 and 11	6 and 12
Basal diet	28.00	28.00	28.00	28.00	28.00	28.00
White corn meal	66.74	50.04	33.37	16.68		
Cereal mix 1.	.	16.90	33.80	50.70	67.52	67.52 [1]
Bone meal	2.86	2.15	1.43	0.72		
Limestone	2.40	2.91	3.40	3.90	4.48	4.48
Totals	100.00	100.00	100.00	100.00	100.00	100.00
Analyses %						
Total Ca	1.60	1.60	1.60	1.60	1.60	1.60
Total P	0.67	0.65	0.64	0.63	0.62	0.79
Non-cereal P	0.67	0.54	0.43	0.31	0.20	0.20
Cereal P	0.00	0.11	0.21	0.32	0.42	0.59
Results						
C.L.O. (Lots 1–6) Body weight Av. 28 days (gm)	268	296	308	303	291	273
Bone ash %	44.6	43.3	40.8	37.0	33.2	34.4
Vitamin D_3 (Lots 7–12) Body weight Av. 28 days (gm)	279	308	325	321	297	304
Bone ash %	45.8	45.3	44.0	40.0	35.6	40.4

[1] Lots 6 and 12 received cereal mixture no. 2, containing 15 parts of corn, 35 parts of wheat middlings and 20 parts of wheat bran. This increased the level of total phosphorus to 0.79% and the cereal phosphorus to 0.59% for lots 6 and 12.

ash is shown graphically in figure 1. Bone ash has been plotted against the logarithm of the per cent non-cereal phosphorus in the diet and a straight line fitted to the data by the method of least squares. This line may be represented by the formula $y = 26.30 + 22.60X$, in which y represents the bone ash expected and X the logarithm of the non-cereal phosphorus,

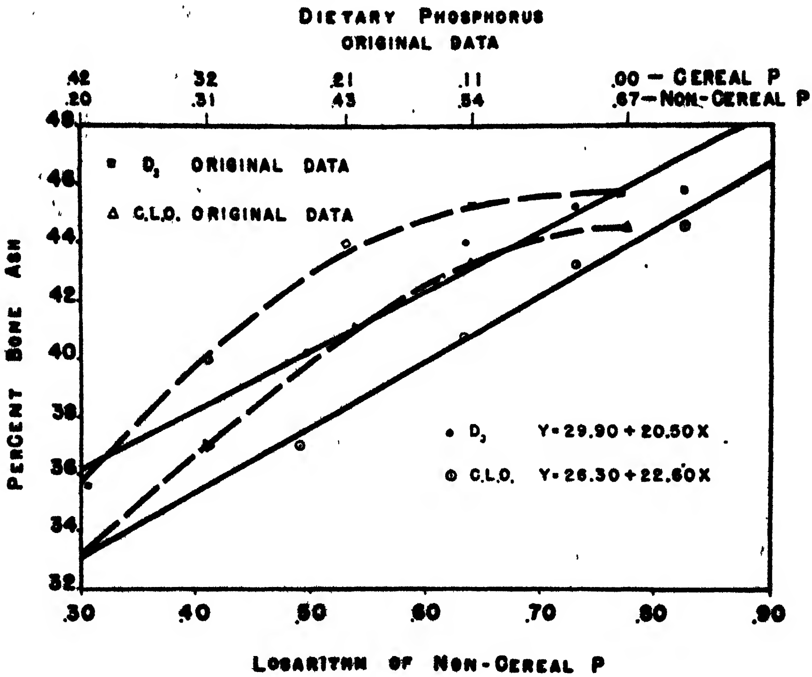


Figure 1

when cod liver oil is the source of vitamin D and $y = 29.90 + 20.50X$ when vitamin D_3 is used. The curved lines represent the original data plotted against the levels of dietary phosphorus actually fed.

In the second experiment, the diets containing non-cereal and cereal phosphorus levels of 0.31 and 0.32, and 0.20 and 0.42%, respectively, were used, since these diets were incapable of supporting normal calcification in experiment 1. Eight lots of day-old poults were placed on each diet, lots 1 to

4 receiving 60, 120, 240 and 480 units of vitamin D per 100 gm of ration, from a pure cod liver oil, and lots 5 to 8 the same unitage from vitamin D_3. The entire experiment was run in duplicate, fresh feed being mixed for each trial. The positions of the lots in the battery were selected with the aid of a randomized block based on a latin square in order to minimize the effect of position in the battery on results. Smaller numbers of poults (6 per lot) were used in this experiment owing

TABLE 3

Summary of the data on experimental rations and results in experiment 2.

LOT NO.	DIETARY PHOSPHORUS	A.O.A.C. CHICK UNITS OF VITAMIN D		RESULTS	
		C.L.O.	D_3	Body wt. at 28 days	Bone ash
				gm	%
1		60		337	37.1
2		120		325	37.2
3	Total phosphorus 0.63%	240		324	38.9
4	Non-cereal P 0.31%	480		328	39.5
5	Cereal P 0.32%		60	326	40.6
6			120	333	42.0
7			240	328	41.9
8			480	344	42.3
9		60		289	32.3
10		120		308	34.9
11	Total phosphorus 0.62%	240		323	33.5
12	Non-cereal P 0.20%	480		288	35.3
13	Cereal P 0.42%		60	286	36.[illegible]
14			120	300	38.8
15			240	314	39.1
16			480	318	39.6

to the fact that it was carried out in duplicate, and also because fewer poults were available at this time. A summary of the experimental data on rations and results appears in table 3. Since the results of the two duplicate series are very similar, they are combined for tabular presentation and statistical analysis.

The data presented in table 3 indicate that increasing the level of vitamin D in the diet will increase the bone ash, but

that the magnitude of the response is small. Increasing the vitamin D from 60 to 480 units per 100 gm of diet (lots 1 to 4) increased bone ash only 2.4% whereas in experiment 1 an increase of 0.12% of non-cereal phosphorus (compare lots 3 and 4) increased bone ash 3.8%. The situation was similar for both sources of vitamin D, although the poults receiving vitamin D_3 always showed better calcification than those receiving cod liver oil. The difference in bone ash obtained with the two sources of vitamin D was dependent, to some extent at least, on the source of the phosphorus in the diet. This can be demonstrated by studying the relationship between the level of vitamin D fed and the per cent bone ash found in the tibia. A line of best fit was calculated by the method of least squares, for the cod liver oil data obtained with each diet, and the number of units of vitamin D from cod liver oil that would be necessary to produce a bone ash equivalent to that obtained at a given level of vitamin D_3 was then computed. The data for lots 1 to 4 may be represented by the formula $y = 36.72 + .006X$ in which y is the per cent bone ash obtained and X the number of A.O.A.C. chick units of vitamin D fed. For lots 9 to 12 the formula is $y = 32.86 + .005X$. By substituting in place of y the bone ash value obtained at any given level of vitamin D_3 one may easily calculate X, the number of units of vitamin D from cod liver oil that would be required to produce that per cent of bone ash. Dividing the number of units of vitamin D required from cod liver oil by the number of units of vitamin D_3 actually fed, gives the efficacy ratio. A summary of the efficacy ratios computed in this manner for the two diets and the several levels of vitamin D used in experiment 2 appears in table 4. It is apparent that the efficacy ratios are wider in every case on the diet containing the larger proportion of cereal phosphorus. In other words as the relative amount of cereal phosphorus (largely phytin) increased, the relative efficiency of the vitamin D_3 compared with the vitamin D of cod liver oil also increased. The rapid decrease in the size of the efficacy ratio with increasing amounts of vitamin D is due to the fact, previously noted, that bone ash

responses were small for relatively large increments of vitamin D. Unless adequate levels of available minerals are present in the diet of the poult, the ability of vitamin D to promote calcification is definitely limited. One may calculate that in experiment 2, 60 units of vitamin D from cod liver oil produced 93.9% of the bone ash obtainable with 480 units and that 60 units of vitamin D_3 produced 95.8% of the bone ash obtainable with 480 units of vitamin D_3. These results certainly demonstrate that the magnitude of the improvement in bone

TABLE 4

Efficacy ratios of vitamin D from C.L.O. to vitamin D_3 on two diets varying in their source of phosphorus. Experiment 2.

A.O.A.C. UNITAGE OF D_3	DIETARY PHOSPHORUS Non-cereal P 0.31% Cereal P 0.32%	DIETARY PHOSPHORUS Non-cereal P 0.20% Cereal P 0.42%
60	10.83 : 1	13.40 : 1
120	7.38 : 1	9.67 : 1
240	3.57 : 1	5.13 : 1
480	1.93 : 1	2.77 : 1
	$y = 36.72 + .006X$	$y = 32.86 + .005X$

y = per cent bone ash; X = A.O.A.C. units of vit. D from C.L.O.

ash that may be expected when the level of vitamin D fed is increased above the minimum requirement, is quite small. Two other facts are brought out by experiment 2. First, on both diets 60 units of vitamin D_3 supported better calcification than did 480 units of vitamin D from cod liver oil, and second, 60 units of vitamin D from either source produced as good calcification in experiment 2 as 80 units of vitamin D from the same sources in experiment 1.

DISCUSSION

That the vitamin D requirements of turkeys, or of all birds and animals for that matter, are intimately associated with the levels of and the ratio between calcium and phosphorus is a generally accepted fact. It is not so well known, however, that the turkey can make little use, for bone calcification, of

much of the phosphorus contained in the cereals and cereal by-products, and that this may influence the vitamin D "requirement" just as much as the total level of this mineral. Certainly the results obtained with lots 1 and 7 in experiment 1 suggest that 80 units of vitamin D per 100 gm of ration is entirely adequate for the turkey. Yet lots 4, 5 and 11 contained several markedly rachitic birds despite the fact that the total mineral and vitamin D intake was essentially the same as that of lots 1 and 7. Increasing the vitamin D to 480 units per 100 gm of feed (expt. 2) eliminated external symptoms of rickets but increased bone ash an average of only 2.45% whereas an increase of 0.11% of non-cereal P (expt. 1, lots 4 and 5, and 10 and 11) increased bone ash 4.10%. The primary problem of bone calcification in the poult appears therefore, to be one of mineral source and level with the logical and most effective solution coming through this channel rather than through manipulation of the vitamin D level.

It has been pointed out by Matterson, Scott and Singsen ('46) and confirmed by the experiments reported here, that many of the discrepancies which appear in the literature as to the vitamin D requirement of turkey poults can be explained in part at least, on the basis of the availability of the phosphorus used in the ration. This relationship is shown clearly by plotting the per cent bone ash against the logarithm of the non-cereal phosphorus in the ration (fig. 1). When the ration contains 0.20 to 0.67% of non-cereal phosphorus, the increase in bone ash is a straight-line function of the logarithm of the non-cereal phosphorus in the diet. If we assume that an average bone ash level of 40%, although far from representing maximum storage of minerals in the bone, represents a minimum practical level of calcification which will prevent rickets, then one may easily calculate from figure 1 that the diet must contain not less than 0.306 and 0.385% non-cereal phosphorus with 80 units of vitamin D per 100 gm of feed from D_3 and cod liver oil, respectively. This assumes, of course, a total phosphorus level of approximately 0.65%. In terms of non-cereal phosphorus the difference between the two

sources of vitamin D is approximately 26% at this level of mineral intake. The percentage difference between the two sources of vitamin D decreases as the level of total non-cereal phosphorus in the diet increases. Although relatively large increases in cereal phosphorus (expt. 1, lots 6 and 12) will promote fair calcification when the diet contains vitamin D_3, this is not true when cod liver oil is used as the source of vitamin D. It is of interest to consider for a moment just what this observation implies. If approximately 75% of the additional 0.17% cereal phosphorus fed lots 6 and 12 (expt. 1) is phytin, then these lots received about 0.04% additional non-phytin phosphorus of cereal origin. While it is conceivable that this amount of available phosphorus could account for the slight increase in bone ash noted between lots 5 and 6, (1.2%) it could scarcely account for the increase noted between lots 11 and 12 (4.8%). This certainly suggests that the action of the vitamin D_3 involved the phytin as well as the non-phytin phosphorus whereas that of cod liver oil probably involved only the non-phytin phosphorus. The results obtained with cod liver oil in these experiments indicate that although small amounts of the phosphorus contributed to the diet by the cereal ingredients may be utilized for calcification, most of it cannot be used for this purpose. This finding is in agreement with the work of Matterson, Scott and Singsen ('46) who used commercial calcium-magnesium phytate, isolated from corn, as the source of dietary phosphorus. Furthermore, the trend of the difference existing between the two sources of vitamin D was similar in both series of experiments. It seems entirely reasonable to conclude, therefore, that the poor utilization of phosphorus by the poult is attributable to the inability of this species to use phytin phosphorus for bone calcification, and that the greater efficacy of vitamin D_3 as compared to cod liver oil is due in part at least to its effect upon the utilization of phytin phosphorus.

Variations in growth in both experiments are relatively small and are not related to variations in the source of phosphorus in the diet. Since it is well known that 0.20% total

phosphorus will neither support life nor growth with turkey poults, it is obvious that the cereal phosphorus, largely in the form of phytin, is being used by the body to carry on the metabolic processes necessary for growth. These findings are in agreement with those of Singsen ('45) for the chick. If decreases in growth do occur in the field, they are probably the result of low feed intake due to rickets and the resultant disinclination to move rather than to metabolic inability to grow. The fact that the birds continue to grow simply aggravates the rachitic condition.

CONCLUSIONS

On the basis of the data presented here, the following conclusions seem justified.

1. Approximately 0.65% of non-cereal phosphorus in the diet will satisfactorily support growth and bone calcification in the poult.
2. Under practical conditions 0.40% non-cereal phosphorus will support adequate calcification and maximum growth with either source of vitamin D, provided the diet contains not less than 0.65% total phosphorus.
3. The vitamin D requirement of the poult, in the presence of adequate available phosphorus and a Ca : P ratio of approximately 2.5 : 1 is not more than 80 and may be less than 60 A.O.A.C. chick units per 100 gm of diet.
4. When the levels of calcium, vitamin D and total phosphorus are held constant, the per cent bone ash decreases with increasing amounts of cereal phosphorus.
5. The phosphorus of a mixture of corn, wheat middlings and wheat bran is relatively unavailable to the poult for bone calcification.
6. The per cent bone ash obtained is a straight line function of the logarithm of the non-cereal phosphorus when the diet contains from 0.20 to 0.67% non-cereal phosphorus, approximately 0.67% total phosphorus, and 80 units of vitamin D per 100 gm of diet.

7. Vitamin D_3 is more effective than the vitamin D from cod liver oil in promoting calcification in poults receiving a diet relatively high in cereal phosphorus and low in non-cereal phosphorus. The difference in efficacy between the two sources of vitamin D decreased as the level of non-cereal phosphorus in the diet increased.

8. The efficacy ratio between vitamin D_3 and the vitamin D of cod liver oil is conditioned by the level and source of phosphorus in the diet as well as the level of vitamin D fed.

9. Increasing the level of vitamin D eight-fold (60 to 480 units) is less effective in improving bone calcification than a 0.11% increase of the non-cereal phosphorus.

10. Cereal phosphorus will support growth which is maximum for the diet and the strain of poults used.

ACKNOWLEDGMENTS

We are indebted to Dr. John Correll of the Upjohn Company for the cod liver oil; Dr. J. Waddell of E. I. DuPont de Nemours and Company for the activated 7-dehydrocholesterol and to Dr. P. L. Harris of the Distillation Products Company for the vitamin A distillate.

LITERATURE CITED

Bird, H. R. 1944 Comparison of response of turkey poults and of chicks to different forms of vitamin D. J. Nutrition, *27:* 377.

Boucher, R. V. 1944 Efficacy of vitamin D from different sources for turkeys. J. Nutrition, *27:* 403.

Evans, R. J., and A. W. Brant 1945 Calcium phosphorus and vitamin D interrelationships in turkey poult nutrition. Poul. Sci., *24:* 404.

Fritz, J. C., J. H. Hooper and H. P. Moore 1945 Calcification in the poult. Poul. Sci., *24:* 324.

Hammond, J. C., H. E. McClure and W. L. Kellogg 1944 The minimum phosphorus requirements of growing turkeys. Poul. Sci., *23:* 239.

Matterson, L. D., H. M. Scott and E. P. Singsen 1946 The influence of sources of phosphorus on the relative efficiency of vitamin D_3 and cod liver oil in promoting calcification in poults. J. Nutrition, *31:* 599.

Mussehl, F. E., and C. W. Ackerson 1935 Calcium and phosphorus requirements of growing turkeys. Poul. Sci., *14:* 147.

SINGSEN, E. P. 1945 The phosphorus requirements of the chicken with special reference to the availability of phytin phosphorus. Ph.D. thesis, Univ. of Ill., Urbana.

SINGSEN, E. P., AND H. H. MITCHELL 1945 Phosphorus in poultry nutrition. I. The relation between phytin and the source of vitamin D. Poul. Sci., *24*: 479.

SOME EFFECTS OF DIETARY ZINC DEFICIENCY IN THE MOUSE[1]

HARRY G. DAY AND BLANCHE E. SKIDMORE
Department of Chemistry, Indiana University, Bloomington

THREE FIGURES

(Received for publication September 19, 1946)

Attempts to produce a deficiency state in mice by means of diets deficient in zinc have been made by Bertrand and Benzon ('22), McHargue ('26), Hubbell and Mendel ('27) and Bertrand and Bhattacherjee ('35). These experiments were unsuccessful either because the diets were not low enough in zinc or they were incomplete in other respects.

Several investigations based on the use of rats have been successful in the production of an unquestionable zinc-deficiency state, as summarized in a recent review (Hegsted, McKibbin and Drinker, '45). If the diet is extremely deficient in zinc growth is markedly impaired (Hove, Elvehjem and Hart, '38); certain specific pathological changes occur (Follis, Day and McCollum, '41) and one enzyme system, the phosphatases, is affected (Hegsted et al., '45).

Although it appears probable that no primary differences occur between rats and mice in respect to the nutritional role of zinc, it is desirable to have direct experimental evidence

[1] Supported, in part, by Grant no. 605 of the Committee of Scientific Research, American Medical Association.

Some of the data in this paper were reported in abstract form in Federation Proceedings, 1942, vol. 1, p. 188.

Taken, in part, from a thesis submitted to the Faculty of the Graduate School by Blanche E. Skidmore in partial fulfillment of the requirements for the Master of Arts degree.

concerning both species. In addition, mice require relatively little food and space.

A logical procedure in attempting to elucidate the nutritional role of zinc is to systematically test the activity of different enzyme systems and hormonal functions in zinc-deficient animals. Schultze and Kuiken ('41) found that catalase, which is an iron porphyrin complex, is much decreased in activity by feeding a copper-deficient diet. In addition, Coulter and Stone ('38) and Kapp ('39) have each reported the isolation of a zinc porphyrin compound from biological material. Therefore, in addition to certain other observations, it was of interest to determine whether a deficiency of zinc would affect the catalase activity of selected tissues.

METHODS

Diet

The preparation and composition of the zinc-deficient diet was the same as described by Day and McCollum ('40) in studies with rats except that the level of the purified casein hydrolysate was 18% and no egg white was used. Owing to the difficulties of determining zinc quantitatively when the concentration is exceedingly low it can only be estimated that the diet contained not more than 0.3 parts per million of zinc, as determined by the Caughey, Holland and Ritchie ('38) method.

The nutritional adequacy of the diet, when supplemented with zinc, was proved by its ability to promote good growth and well being over long periods of time.

Care of the animals

Mice of the CHI strain [2] were used throughout. Individual litters, with their mothers, were removed from the stock breeder cages when they were 16–18 days old and placed in zinc-free cages containing acid-washed filter paper. The zinc-

[2] The breeding stock was obtained from Dr. L. C. Strong of the Yale University School of Medicine.

low diet was furnished. After 7 to 10 days the mother was returned to the stock colony and the litter was divided as equally as possible with regard to weight and sex.

The cages consisted of 10-liter glazed stone jars each containing a 3-mesh monel metal floor raised 1 inch above the bottom of the jar. Small pyrex glass food cups and drinking tubes were used. The water was distilled in pyrex glass. The cages were washed frequently and rinsed with hot zinc-free water. Contamination of the animals by dust and other possible extraneous sources of zinc was avoided as much as possible by keeping them in a room reserved for that purpose.

Zinc, as zinc sulfate, was given to the control animals each day by pipetting 0.2 ml of an aqueous solution onto the food. This furnished 40 µg of zinc per mouse.

Determination of catalase activity

Zinc-deficient mice and their controls were lightly anaesthetized by ether and decapitated. Two 0.1 ml aliquots of the shed blood were each pipetted immediately into 10.0 ml of redistilled water. These stock dilutions were further diluted immediately before making the catalase determinations.

The liver and kidneys were removed immediately, rinsed, blotted with ash-free paper, weighed, and ground to a thin paste in a zinc-free mortar. Then each tissue was diluted so that 1 ml contained 10 mg of tissue. After being refrigerated for about 10 hours the samples were centrifuged and the supernatant liquids were used for catalase determinations. The kidney supernatant was used without further dilution but the liver was diluted again, 4 ml of liver supernatant to 6 ml of water.

Catalase determinations were made in accordance with the method of Schultze and Kuiken ('41) except that the reaction interval was 6 minutes instead of 2. The catalase activity of liver and kidney was expressed in terms of the nitrogen content of the samples analyzed, instead of the dry weight. Greenstein et al. ('42) and some others have followed this

practice. Therefore, all the diluted liver and kidney samples were analyzed for total nitrogen, using a conventional micro-Kjeldahl method.

RESULTS

Growth and survival

About 170 mice were used in experiments which resulted in significant growth data. Impairment of growth in young mice on the zinc-deficient diet became manifest within 2 weeks. After 5 to 7 weeks surviving animals had practically ceased to grow. This was usually followed by loss of weight and eventual death. Figure 1 illustrates the effects on growth.

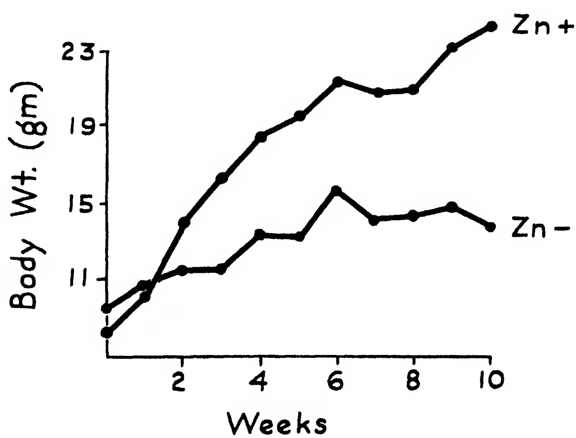


Fig. 1 Effect of dietary zinc deficiency upon the growth of young male mice. The growth of females is qualitatively the same.

Approximately 18% of the animals on the zinc-deficient diet failed to survive more than 8 weeks. This figure is based on the records from 125 mice. The survival of controls (45 animals) was 100%. Most of the fatalities occurred between the third and fifth weeks.

Within the first 2 to 3 weeks the zinc-deficient mice showed signs of progressive emaciation; the coat became greasy and unkempt; and loss of hair from the top of the head, neck and shoulders occurred in approximately 50%. Whether the

alopecia was directly related to the zinc deficiency is regarded as questionable. It may be significant that the affected parts were only those that can not be licked by the animals. The general appearance of the mice is illustrated in figure 2. Supplementation of the diet with inositol (0.5 mg per mouse per day) did not affect the alopecia.

Analyses of the bones (femurs and tibia-fibulas) by standard methods (Day and Follis, '41) showed that the percentage

Fig. 2 The two male mice are littermates. They were on the zinc-deficient diet 28 days, but the lower one received 40 μg of zinc per day added to the food as zinc sulfate.

of bone ash was not changed by zinc deficiency although the total weight of ash was markedly reduced, as shown in table 1.

Also, the growth of the liver and kidneys was correspondingly decreased by the deficiency (table 1). All the evidence indicated that the lack of zinc had a generalized effect on growth and that the element probably is essential in many, if not all, structures of the body.

TABLE 1

Effect of zinc deficiency on the mineralization of bone and the growth of liver and kidneys.

NO. MICE AND SEX	DAYS ON DIET	LIVER	KIDNEY	BONE[1]	
				Ash	% Ash
		gm	*gm*	*mg*	
		Zinc-deficient			
10 M	30	0.69	0.16	37.8	50.2 ± 2.3[2]
6 F	31	0.74	0.17	38.5	52.2 ± 4.1
		Zinc-added			
9 M	30	1.27	0.26	47.5	48.9 ± 2.7
5 F	31	1.30	0.22	45.3	48.9 ± 4.8

[1] Femurs, tibias and fibulas combined.

[2] Average deviation of a single determination from the arithmetical mean.

Effects of cadmium

Owing to the close chemical similarities between zinc and cadmium tests were made to determine the effects of the latter on the growth of zinc-deficient mice. The dosage of cadmium, as cadmium sulfate, was 4 µg per mouse per day. The element was without ability to substitute for zinc, as indicated by the growth data from 21 animals.

Catalase

Table 2 summarizes the results of the catalase determinations. The catalase activity of liver and kidney was markedly decreased in both males and females on the zinc-deficient diet, as shown by comparison with controls given the same diet with zinc added and by comparison with others on a stock

diet. This difference was substantially the same whether expressed in terms of tissue nitrogen, dry weight or wet weight. The blood catalase activity was not changed.

In a number of cases the effect of added zinc, as zinc sulfate, was determined. The results, given in figure 3, represent the

TABLE 2

Effects of zinc deficiency on the catalase activity of mouse liver, kidneys and blood.

DIET	NO. MICE AND SEX		DAYS ON DIET	WT. GAIN WHILE ON DIET	LIVER	KIDNEYS	BLOOD
					ml 0.02 N H_2O_2 decomposed per		
					mg N	mg N	ml blood
				gm			
Zn —	14	M	27	1.7	95	129	3037
Zn +	15	M	27	9.8	187	174	3133
Stock	4	M			170	178	3247
Zn —	10	F	26	1.8	77	74	2916
Zn +	4	F	26	10.5	149	90	2790
Stock	3	F			140	120	3178

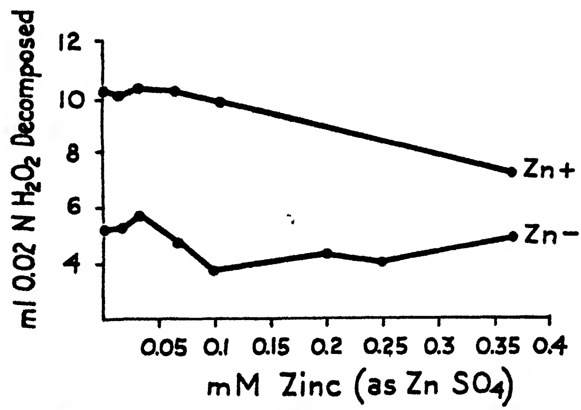


Fig. 3 The effect of zinc, added as zinc sulfate, on the catalase activity of liver from zinc-deficient mice and from controls receiving zinc in the diet.

general findings when liver is used. There was no appreciable change in the liver catalase activity either in zinc-deficient or control mice when zinc sulfate was added in amounts ranging from 0 to 0.37 mm of zinc. Considerably larger concentrations of zinc caused a slight decrease in the activity.

The large sexual difference with respect to the catalase activity of liver and kidney, shown in table 2, confirms the results reported by Schultze and Kuiken ('41). The values were highest in the males.

Liver esterase

No changes were found in the activity of liver esterase, as determined by the method of Harrer and King ('41). The average value for liver from zinc-deficient mice was 0.81, expressed as ml of 0.01 N alkali per mg of dry tissue, per 10 minutes. The average for control animals was 0.77. The activity of this enzyme decreases in ascorbic acid deficiency (Harrar and King, '41) and in some other deficiency states.

Riboflavin [3]

Owing to corneal changes which appeared to simulate those seen in riboflavin deficiency (Follis et al., '41) it was believed that a deficiency of zinc might affect the metabolism of riboflavin and that changes in the concentration of this vitamin in the tissues might occur. However, the riboflavin content of liver and kidney of zinc-deficient mice, as determined by microbiological assay (Snell and Strong, '39) was the same as in the controls.

Teeth [4]

Histological examinations were made of the teeth from zinc-deficient mice and their controls.[5] Over 20 animals were examined. Four of the mice had been on the zinc-deficient diet 89 days. Although weanling mice invariably failed to survive that long on the diet these 4 were exceptions. They were 37 days old before being restricted to the zinc-deficient diet.

[3] The riboflavin determinations were made by Dr. Kenneth P. Broshears and Dr. L. S. McClung to whom we are indebted.

[4] The authors deeply appreciate the help Dr. Schour rendered in making this part of the investigation possible.

[5] This was done by Dr. Isaac Schour of the University of Illinois College of Dentistry. The animals were killed at Bloomington and preserved in formalin before being sent to Dr. Schour.

No changes in enamel or dentin were found in either the incisors or molars. The average length of time on the experimental diets was approximately 47 days. All mice deprived of zinc were extremely deficient as indicated by their appearance, poor growth, and the catalase activity of their livers and kidneys.

DISCUSSION

The observations recorded here, in addition to those elsewhere concerning the effects of zinc deficiency upon the rat (Hegsted et al., '45) demonstrate the essentiality of zinc for higher animals. The results show liver and kidney catalase must now be added to the very small list of enzyme systems known to be affected by this deficiency. However, the investigations to date have not revealed any biochemical lesions or pathological effects of such magnitude that they would seem to be fully accountable for the baneful effects of extreme zinc deficiency.

Debilitating conditions in general do not all affect the catalase activity of tissues. Our unpublished results with rats made extremely deficient in folic acid by the addition of succinylsulfathiazole to a purified diet has shown that the catalase activity of liver, kidney and blood is not decreased. Lepkovsky and Parsons ('43) found no decrease in the activity of this enzyme in pyridoxine-deficient rats. Also, starvation up to 3 days does not affect liver catalase activity (Greenstein et al., '42).

Some instances of marked differences between tissues with respect to the degree of change in catalase activity may be found in the literature. Schultze and Kuiken ('41) reported that the catalase of blood, as well as that of liver and kidney, underwent a marked decrease in activity when the animals were deficient in either copper or iron. However, the activity of catalase in the ventricles was increased in copper deficiency. Greenstein et al. ('42) observed no decrease in blood catalase activity of tumor-bearing mice and rats even though the activity of the enzyme in the liver and kidneys was greatly decreased.

The function of zinc in affecting catalase must be regarded as indirect because the addition of zinc salts to tissue preparations from zinc-deficient mice does not increase the catalase activity. Further evidence in this direction is the fact that none of the crystalline catalase enzymes investigated have been found to contain zinc.

No evidence is available to explain the decrease in catalase activity. It might be due to a general decrease in the rate of production; but if this were true the activity of blood catalase should also decline. Another consideration is that the catalase in liver and kidney is not the same as that in blood. Some support of this hypothesis is afforded by Sumner, Dounce and Frampton's ('40) discussion of the possible occurrence of several catalases differing in activity which, it is suggested, might "result from enzyme action in the liver (or kidneys) of an animal before or after death." On this basis it is possible that the catalase in the liver and kidneys of the zinc-deficient mice consisted of less active enzyme components than the catalase in corresponding tissues of the control animals. If sufficient quantities of tissue material from zinc-deficient animals could be secured it would be interesting to test this hypothesis by attempting to concentrate the enzyme(s) and compare the "Kat.f." of such catalase with that from control animals.

SUMMARY

Weanling mice on an extremely zinc-deficient diet were greatly retarded in the rate of growth. A considerable percentage failed to survive more than 8 weeks. The animals became emaciated, lost hair from the shoulders and back of the neck, but no truly distinctive gross symptoms were noted. This is regarded as evidence that in nutrition zinc has diverse functions.

The catalase activity of the liver and kidneys was markedly reduced. Blood catalase was not affected. The addition of zinc salts to the tissue preparations did not appreciably affect the catalase activity.

No changes occurred in liver esterase and in the concentration of riboflavin in liver and kidneys.

Histological investigations of the teeth failed to reveal any evidence of change attributable to the lack of zinc.

LITERATURE CITED

BERTRAND, G., AND B. BENZON 1924 Recherches sur l'importance du zinc dans l'alimentation des animaux. Experiences sur la souris. Bull. Soc. chim. biol., *6*; 203.

BERTRAND, G., AND R. C. BHATTACHERJEE 1935 Recherches sur l'action combinée du zinc et des vitamines dans l'alimentation des animaux. Bull. Soc. chim. biol., *17*; 1137.

CAUGHEY, R. A., E. B. HOLLAND AND W. S. RITCHIE 1938 Report on zinc. J. Assoc. Off. Agric. Chem., *21*; 204.

COULTER, C., AND F. M. STONE 1938 Identification of the porphyrin compound found in cultures of C. diphtheriae and mycobacteriae. Proc. Soc. Exp. Biol. Med., *38*; 423.

DAY, H. G., AND R. H. FOLLIS, JR. 1941 Skeletal changes in rats receiving estradiol benzoate as indicated by histological studies and determinations of bone ash, serum calcium and phosphatase. Endocrinology, *28*; 83.

DAY, H. G., AND E. V. MCCOLLUM 1940 Effects of acute zinc deficiency in the rat. Proc. Soc. Exp. Biol. Med., *45*; 282.

FOLLIS, JR., R. H., H. G. DAY AND E. V. MCCOLLUM 1941 Histological studies of the tissues of rats fed a diet extremely low in zinc. J. Nutrition, *22*; 223.

GREENSTEIN, J. P., W. V. JENRETTE AND J. WHITE 1942 The liver catalase activity of tumor-bearing rats and the effect of extirpation of the tumors. J. National Cancer Inst., *2*; 283.

HARRER, C. J., AND C. G. KING 1941 Ascorbic acid deficiency and enzyme activity in guinea pig tissues. J. Biol. Chem., *138*; 111.

HEGSTED, D. M., J. M. MCKIBBIN AND C. K. DRINKER 1945 The biological hygienic, and medical properties of zinc and zinc compounds. Suppl. no. 179. Pub. Health Rep., 44 pp.

HOVE, E., C. A. ELVEHJEM AND E. B. HART 1938 Further studies on zinc deficiency in rats. Am. J. Physiol., *124*; 750.

HUBBELL, R. B., AND L. B. MENDEL 1927 Zinc and normal nutrition. J. Biol. Chem., *75*; 567.

KAPP, E. M. 1939 Spectographic observations on urinary porphyrins; the nature of a combined form of coproporphyrin found in urine. Brit. J. Exp. Path., *20*; 33.

LEPKOVSKY, S., AND D. PARSONS 1943 The effects of pyridoxine deficiency in the rat upon the catalase activity of its tissues. J. Biol. Chem., *149*; 281.

McHargue, J. S. 1926 Further evidence that small quantities of copper, manganese and zinc are factors in the metabolism of animals. Am. J. Physiol., *77;* 245.

Schultze, M. O., and K. A. Kuiken 1941 The effect of deficiencies in copper and iron on the catalase activity of rat tissues. J. Biol. Chem., *137;* 727.

Snell, E. E., and F. M. Strong 1939 A microbiological assay for riboflavin. Ind. Eng. Chem., Anal. Ed., *11;* 346.

Sumner, J. B., A. L. Dounce and V. L. Frampton 1940 Catalase. III. J. Biol. Chem., *136;* 343.

SEASONAL VARIATIONS IN THE VITAMIN A AND THE CAROTENE CONTENT OF RETAIL BUTTERS [1]

H. A. ELLENBERGER, N. B. GUERRANT AND O. B. FARDIG
The Pennsylvania State College, State College

THREE FIGURES

(Received for publication August 27, 1946)

Early in 1941 the Office of Experiment Stations in Washington, D. C., invited the State Agricultural Experiment Stations to cooperate in a nation-wide study relative to the carotene and the vitamin A content of butters produced throughout the country. To date, reports have been published by the following collaborating laboratories: Wisconsin (Berl and Peterson, '43); Minnesota (Jenness and Palmer, '45); U. S. Dept. Agric., '45; and Kansas (Parrish, Martin, Atkeson and Hughes, '46).

The study described in the present paper was a part of the national cooperative project and was designed to yield information regarding seasonal fluctuations in carotene and in vitamin A in typical market butters as purchased on the Pennsylvania retail market.

EXPERIMENTAL

Collection of samples

The butters used in this study consisted of 5 nationally-advertized brands and 1 brand produced by a local

[1] Authorized for publication on August 22, 1946, as paper No. 1338 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

creamery. The nationally-advertized butters were purchased on the retail market while the product of the local creamery was purchased directly from the creamery. In general, the samples were purchased semi-monthly. Whenever possible, 1 sample of each product was purchased in the first half and the second sample in the last half of each month. Because of the limited available supply of butter during certain portions of the period of investigation, it was not always possible to obtain 2 samples of each brand of butter in a given month. To make the study more complete, samples of the respective butters which were not available during the current year were purchased during the corresponding weeks and months of the following year. The investigation, therefore, extended for 2 years, from October 1943 to October 1945.

All samples of butter were scored for quality by representatives of the Division of Dairy Manufacturing previous to being assayed. The butters, on being received at the laboratory, were allowed to stand at room temperature until sufficiently warm to permit thorough mixing. The mixing was conducted in a porcelain evaporating dish by means of a porcelain spatula. The butter samples were then placed in airtight glass jars and stored in a refrigerator at 5°C. until they could be assayed.

Methods of analysis

All samples were analyzed for vitamin A and for carotene by the procedures described by Koehn and Sherman ('40) after certain modifications as recommended by the Technical Committee appointed for the National Project by the Agricultural Research Administration. These methods have been outlined in considerable detail by Jenness and Palmer ('45). Since the sources of some of the butter samples were unknown, 92% methanol was used throughout the investigation to extract the noncarotene pigments. Light transmission measurements were made by means of an Evelyn photoelectric colorimeter, using a 620 mμ filter for vitamin A measurements

and a 440 mμ filter for establishing the vitamin A correction factor and for beta carotene measurements. Concentrations of vitamin A and carotene were determined by use of standard reference curves prepared with crystalline vitamin A alcohol and with crystalline beta carotene.

DATA

The results of this investigation are presented in condensed form in figures 1–3, inclusive.

DISCUSSION

Methods of analysis

While carotene and vitamin A were determined by the assay procedures indicated above, certain tests were made during the course of the investigation for the purpose of ascertaining the possible effects of certain variations in laboratory procedures, reagents, etc., on the results of the assay. The results of some of these observations appear to merit mention.

Reports have appeared in the literature relative to the merits of 92% methanol or of 94% diacetone alcohol in the phasic separation and the use of a chromatographic procedure for separating the noncarotene pigments from beta carotene when the carotene is dissolved in petroleum ether or in Skellysolve (Berl and Peterson, '43; Hauge, Westfall, Wilbur and Hilton, '44; and Zscheile, Nash, Henry and Green, '44). Investigations carried out along this line, using a limited number of samples of butter, revealed that when the solutions were extracted with 94% diacetone alcohol approximately 11% less pigment remained than when the extraction was made with 92% methanol (for example, 4.71 μg of carotene per gm as against 5.31 μg per gm). Furthermore, in the chromatographic procedure, where dicalcium phosphate was used as the adsorbing agent, 10% less pigment remained than when the extraction was made with 92% methanol (for example, 5.56 μg per gm against 6.17 μg per gm). While it cannot be said that these findings will apply to all types of butters,

these results are in general agreement with those reported by the above investigators.

Some observations were made regarding the effect of using antimony trichloride reagent of variable degrees of freshness. In the studies herein reported the reagent was always stored in a brown, glass-stoppered bottle in a dark compartment of the laboratory desk. After several months of storage, it was found that the reagent yielded results comparable with those obtained with the freshly-prepared reagent. This is contrary to the findings of Edisbury ('40) but agrees with the findings of Dann and Evelyn ('38) and those of Benham ('44). In fact, it was observed that when the residual solution and undissolved crystals from several bottles of the reagent were combined in 1 bottle over a period of months, the combined reagents yielded vitamin values which agreed with those obtained when the freshly-prepared reagent was employed. However, as pointed out by Dann and Evelyn ('38), when the older reagent was used the blue color was observed to fade more rapidly.

Peroxides, frequently present in diethyl ether, were found to have a marked deleterious effect on vitamin A but appeared to have no significant effect on carotene. This is illustrated by the data presented in table 1.

TABLE 1

Effect of using diethyl ether containing traces of peroxides in the analysis of butters for vitamin A and carotene.

SAMPLE NUMBER	CONCENTRATION (μG PER GM)			
	Vitamin A		Beta carotene	
	Peroxide-containing ether	Peroxide-free ether	Peroxide-containing ether	Peroxide-free ether
126	6.22	9.82	5.12	5.12
127	4.16	7.50	6.17	6.17
128	4.62	7.44	6 23	6.44
129	6.34	9.40	6 87	6.70
130	7.32	9.48	6.17	5.97
131	6.22	8.94	7.92	7.85
133	5.38	9.74	4.74	4.89

Investigations carried out for the purpose of determining the per cent recovery of vitamin A alcohol and beta carotene, when added to butter, gave somewhat varying results, but in general indicated that the losses were negligible. While small losses of added vitamin A have been reported by other investigators (Zscheile, Henry, White, Nash, Shrewsbury and

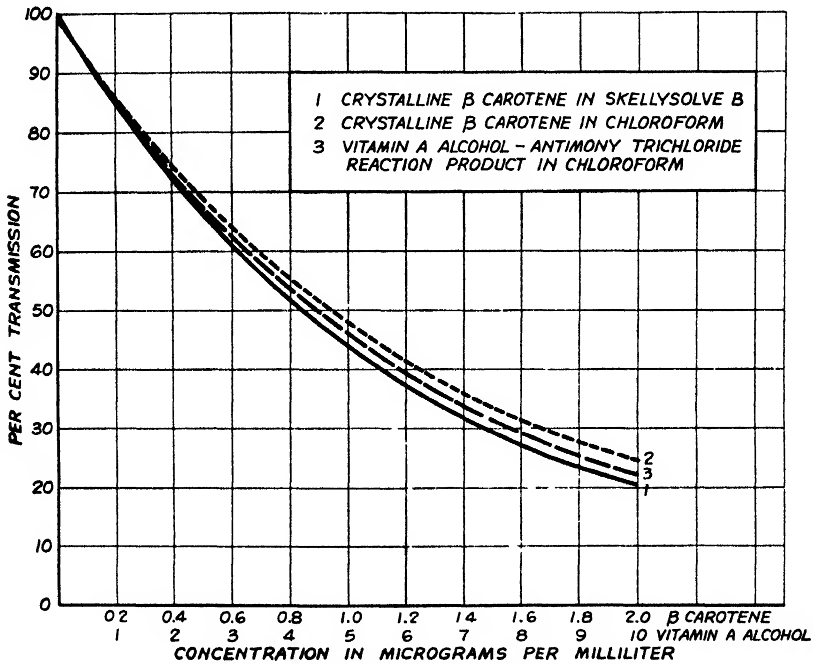


Fig. 1 Concentration curves used in evaluating the carotene and the vitamin A content of butters. The concentration of beta carotene was measured while using a 440 mμ filter and that of vitamin A-antimony trichloride reaction product with a 620 mμ filter.

Hauge, '44; and Jenness and Palmer, '45), the results reported in this publication have not been corrected for loss of vitamin during analysis inasmuch as no appreciable loss was detected.

The standard curves used in evaluating the vitamin values reported at this time are given in figure 1. The K values for

these curves when calculated between 30 and 80% transmission were 14.86 for vitamin A alcohol-antimony trichloride reaction product, 2.77 for beta carotene in Skellysolve B, and 3.15 for beta carotene in chloroform, where K equals concentration in micrograms per milliliter divided by 2–log G (Galvanometer reading). Slightly different standard curves were used in evaluating the potencies of butters reported in a previous publication (U. S. Dept. Agric. Misc. Pub. No. 571, '45).

Before evaluating accurately the probable nutritional value of butter from its reported vitamin A and carotene content, one must recognize the following sources of error: (a) lack of complete separation of beta carotene from contaminating pigments, (b) lack of a satisfactory vitamin A standard in terms of which biological values may be expressed, and (c) lack of definite conversion factors which may be used to convert physical measurement data into biological units. Some of the difficulties encountered in attempts to separate beta carotene quantitatively from contaminating pigments by phasic separation and by chromatographic procedures have been indicated above. In addition to existing limitations, neither of these procedures is expected to separate quantitatively beta carotene from alpha carotene, cryptoxanthin and other carotenoids having lower biological values. Gridgeman ('44) has reviewed much of the published literature relative to the variability of the proposed vitamin A and carotene standards as well as that relating to some of the suggested conversion factors. The authors have determined the extinction coefficients of numerous samples of what was supposed to be pure crystalline vitamin A alcohol and pure crystalline beta carotene as purchased from supply houses. The results of these measurements showed $E^{1\%}_{1\,cm}$ at 328 mμ ranging from 1320 to 1990 for vitamin A alcohol and $E^{1\%}_{1\,cm}$ at 450 mμ from 2000 to 2590 for beta carotene. The differences in the extinction coefficients of the contents of vials having the same control number was invariably less than the differences between vials having different control numbers.

The vitamin A values herein reported have been corrected for the presence of carotene in the test solution by applying the correction factor 0.007 as directed by the Technical Committee on Methods for this general project. However, when solutions of beta carotene in chloroform, ranging in concentration from 10 to 22 μg per ml, were prepared and the effect of the antimony trichloride reagent tested as suggested, the results indicated a correction factor of 0.004. Berl and Peterson ('43) had previously reported the correction factor to be 0.007, while Parrish, Martin, Atkeson and Hughes ('46) have more recently reported this factor to be 0.005. However, any correction factor determined as indicated above does not take into consideration any $SbCl_3$-reaction product that may be formed by some of the pigments other than beta carotene known to be present in butter. Furthermore, the presence of different amounts of these pigments in butter would be expected to lead to correction factors different from those indicated above. The authors are of the opinion that errors in assay values arising from these sources are also worthy of consideration.

Results of butter analysis

The seasonal variations in the vitamin A and carotene content of butters purchased on the local retail market, as demonstrated by the examination of 142 samples over a period of 2 years, are shown by the data presented in figure 2. In this figure, the five nationally-advertized butters are indicated by numbers 1–5, inclusive, while the butter from the local creamery is designated as butter number 6. An examination of these data as they are related to butter score indicated that there was no apparent relationship between the usual quality score and the vitamin content of the respective butters. These observations are in agreement with those of Berl and Peterson ('43) and also those of Parrish, Martin, Atkeson and Hughes ('46).

Some interesting generalizations concerning the relationship and the variability of vitamin A and carotene in the

nationally-advertized butters may be drawn as the result of further examination of the data presented in figure 2. It is found that the average vitamin A content of the 5 butters during the months of January–June, inclusive, was 0.526 mg

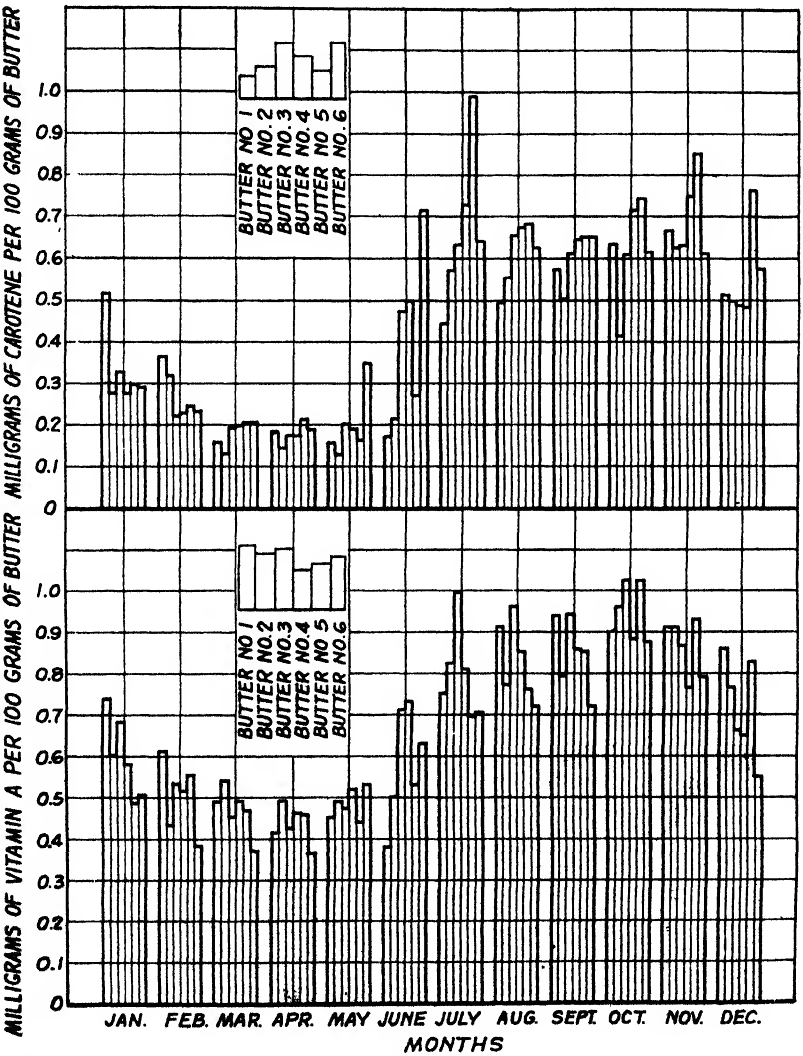


Fig. 2 Seasonal variations in the carotene and the vitamin A content of butters as purchased on the retail market.

per 100 gm of butter while the average vitamin A content of the same butters during the months of July–December, inclusive, was 0.857 mg per 100 gm. Accordingly, "summer–fall" butters contained an average of 63% more vitamin A than "winter–spring" butters. Likewise, it is found that the average carotene content of the 5 nationally-advertized butters during the months of January–June, inclusive, was 0.244 mg per 100 gm of butter, whereas the average carotene content of the same butters for the remaining 6 months of the year was 0.625 mg per 100 gm. These "summer–fall" butters therefore contained an average of 165% more carotene than the corresponding "winter–spring" butters. When one considers 1 μg of vitamin A equivalent to 4 I.U. and 1 μg of the above carotene equal to 1.67 I.U., these data reveal that the average vitamin A potency of these butters exceeded the carotene equivalent during the months of January–June, inclusive, by 416%, whereas it only exceeded the carotene equivalent by 228% during the remaining months of the year. In addition, the vitamin A content of these butters remained somewhat more constant throughout the year than did the carotene content. This is in agreement with the findings of others (Berl and Peterson, '43; Jenness and Palmer, '45; Lord, '45; and Parrish, Martin, Atkeson and Hughes, '46). As would be expected, similar observations have been reported for vitamin A in bovine blood plasma (Sutton and Soldner, '45; and Lord, '45).

While the data show that there were measurable differences in the vitamin A and in the carotene content of different butters during a given month, the average vitamin values for the respective months indicate a definite seasonal variation in vitamin content. Intraseasonal variations in vitamin A and carotene content have been reported by Berl and Peterson ('43), Jenness and Palmer ('45), and Parrish, Martin, Atkeson and Hughes ('46). In the present studies, however, there were no indications that any one butter was continually higher or continually lower in either vitamin A or carotene than other butters under investigation.

The data presented in figure 3 are based on the previous assumption that one International Unit of vitamin A is equivalent to the biological effect of 0.25 µg of vitamin A alcohol (Berl and Peterson, '43) or to 0.6 µg of beta carotene (Quart. Bull. Health Organization, League of Nations 4, 431,

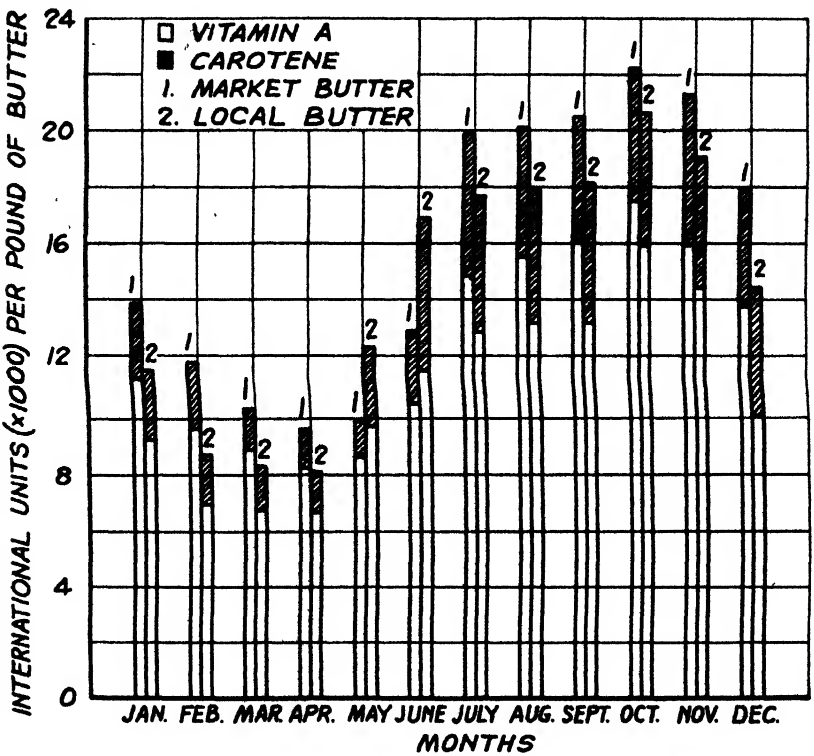


Fig. 3 Seasonal variations in the contribution that carotene and vitamin A made to the biological potency of butters. The values given for the market butter are the average values obtained for the 5 nationally advertized butters.

1934). However, biological assays conducted in this laboratory do not substantiate this relationship for vitamin A alcohol, the alcohol being somewhat less potent than the above figure would indicate. The butter purchased from the local creamery had the lowest vitamin potency during the months of February, March and April with the potency showing a

rapid rise during May and June, with further but more gradual increases in potency during July, August and September. The highest potency occurred in October. The 5 commercial butters showed the lowest potency during the months of March, April and May with the potency rising rapidly during June and July, a tapering off in the rate of increase during August and September, with the highest potency likewise occurring in October. Perhaps it should be expected that these butters would reach their peak in vitamin potency in November, considering the delay observed in the rate of vitamin enrichment during the spring months. Although the data show that the biological potencies of the butters increased during the months of June, July, August and September, with the highest potency occurring in October, the data also reveal that the amount of potency attributable to carotene was somewhat less during August and September than during either July or October. Perhaps this reflects the carotene content of the dairy ration.

The average vitamin A potency of the 6 brands of butter for the year was found to be 15,640 I.U. per pound. Considering the possible differences in standards used, this value compares favorably with those reported in the literature. Of this potency, 12,290 I.U. were calculated as having been contributed by vitamin A, while 3,350 I.U. were contributed by carotene.

The data regarding seasonal variation in the vitamin A potency of the butter purchased at the local creamery are in agreement with the data reported elsewhere concerning similar butters (Jenness and Palmer, '45; Lord, '45; and Parrish, Martin, Atkeson and Hughes, '46). Inasmuch as the over-all seasonal variation in the vitamin potency of the commercial butters paralleled the variations in the vitamin potency of the fresh butter obtained from the local creamery, these data indicate that, during the period of this study, commercial butters moved from manufacturer to consumer without delay. When possible differences in the vitamin standards used by different investigators are taken into consideration, these data further reveal that the total vitamin A potencies

of the 5 commercial butters herein reported compare favorably with those values reported for butter taken at the point of manufacture. This would tend to show that no significant loss in vitamin potency takes place during the normal period of time required for butter to pass from the manufacturer to the consumer. The present findings regarding this matter are in full agreement with the summarized results thus far reported by collaborators participating in the national butter project.

SUMMARY

A study of the seasonal variations in the vitamin A and carotene of commercial butter as purchased on the Pennsylvania market is described. In the course of a 2-year period more than 140 samples of butter were assayed. These samples represented 5 nationally-advertized brands of butter purchased on the retail market and 1 brand purchased at a local creamery.

Preliminary to and during the course of these assays, studies relating to methodology were carried out for the purpose of checking or improving methods of assay. The results of these studies are summarized as follows:

1. Freshly-prepared solutions of the antimony trichloride reagent and solutions which had been prepared for several months yielded essentially the same vitamin A values when used with the same butters.

2. In the analysis of butter, the 94% diacetone alcohol procedure and the chromatographic procedure for eliminating the noncarotene pigments yielded lower carotene values than did the procedure in which 92% methanol was used.

3. Peroxides, frequently found in diethyl ether, were found to exert a marked deleterious effect on vitamin A stability but appeared to have no effect on carotene stability during the usual course of the assay.

4. Some of the vitamin A standards and carotene standards in use at the present time were found to vary with respect to their extinction coefficients.

5. Although distinct seasonal variations in the carotene and vitamin A content of butters as purchased on the retail market in Pennsylvania were noted, there was no indication that any one brand of butter was constantly higher or constantly lower than other brands with respect to either vitamin A or carotene.

6. With few exceptions, the vitamin A content of butter was found to exceed the carotene content (in terms of both weight of material present and International Unit equivalent) at all seasons of the year and also showed less seasonal variation.

7. The average yearly vitamin potency of the 6 brands of butter was found to be 15,640 I.U. per pound.

8. A comparison of the seasonal vitamin A potencies of the commercial butters with those of the butter from the local creamery and with vitamin values reported in the literature for fresh butters indicated that during the course of this study the vitamin content of butter was relatively stable and that not much time elapsed from the date of manufacture until the butter reached the consumer.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Professor I. E. Parkin of the Division of Dairy Husbandry Extension for collecting the samples of butter used in the early part of the investigation and to Professors F. J. Doan and D. V. Josephson of the Division of Dairy Manufacturing for scoring the numerous samples of butter used throughout the investigation.

LITERATURE CITED

BENHAM, G. H. 1944 Antimony trichloride method for determination of vitamin A. Canad. J. Res., *22:* Sec. B, 21.

BERL, S., AND W. H. PETERSON 1943 Determination and content of carotene and vitamin A in Wisconsin butter. J. Nutrition, *26:* 527.

DANN, W. J., AND K. A. EVELYN 1938 The determination of vitamin A with the photoelectric colorimeter. Biochem. J., *32:* 1008.

EDISBURY, J. R. 1940 Spectrophotometric assay of vitamin A with special reference to margarine. Analyst, *65:* 484.

GRIDGEMAN, N. T. 1944 The Estimation of Vitamin A. Lever Brothers and Unilever Limited.

HAUGE, S. M., R. J. WESTFALL, J. W. WILBUR AND J. H. HILTON 1944 The vitamin A requirements of dairy cows for production of butterfat of high vitamin A value. III. Availability of carotene in dehydrated alfalfa hay as compared with carotene in oil. J. Dairy Sci., *27:* 63.

JENNESS, R., AND L. S. PALMER 1945 The vitamin A potency of creamery butter produced in Minnesota. J. Dairy Sci., *28:* 473.

KOEHN, C. J., AND W. C. SHERMAN 1940 The determination of vitamin A and carotene with the photoelectric colorimeter. J. Biol. Chem., *132:* 527.

LORD, J. W. 1945 Seasonal variation of carotene and vitamin A in butterfat and in serum. Biochem. J., *39:* 372.

PARRISH, D. B., W. H. MARTIN, F. W. ATKESON AND J. S. HUGHES 1946 The vitamin A and carotene content of market butter produced in Kansas. J. Dairy Sci., *29:* 91.

SUTTON, T. S., AND P. A. SOLDNER 1945 Seasonal variations in the blood plasma carotene and vitamin A of adult dairy cattle. J. Dairy Sci., *28:* 859.

U. S. DEPARTMENT OF AGRICULTURE 1945 Vitamin A in butter. Misc. Pub. No. 571.

ZSCHEILE, F. P., R. L. HENRY, J. W. WHITE, H. A. NASH, C. L. SHREWSBURY AND S. M. HAUGE 1944 Determination of vitamin A and carotenoids in butterfat. Spectroscopic characteristics of butterfat fractions and problems involved in biological interpretations. Ind. and Eng. Chem., Anal. Ed., *16:* 190.

ZSCHEILE, F. P., H. A. NASH, R. L. HENRY AND L. F. GREEN 1944 Determination of vitamin A and carotenoids in butterfat. Comparison of direct spectrophotometry with filter photometry and use of the antimony trichloride reaction. Ind. and Eng. Chem., Anal. Ed., *16:* 83.

ABSORPTION AND STORAGE OF VITAMIN A IN THE LIVER OF THE RAT

JANET M. LEMLEY, R. A. BROWN, O. D. BIRD AND A. D. EMMETT
The Research Laboratories, Parke, Davis and Co., Detroit, Michigan

(Received for publication June 7, 1946)

It has long been recognized that the animal body is capable of accumulating vitamin A and that the liver is the chief storage site (Osborne and Mendel, '18). Studies regarding the distribution of stored vitamin A in the rat's body have shown that 90 to 95% is found in the liver (Sherman and Boynton, '25; Moore, '31; McCoord and Luce-Clausen, '34) and that the liver content of the vitamin is usually proportional to the intake (Baumann, Riising and Steenbock, '34). The liver stores of the vitamin may indicate both the state of nutrition of the animal and the availability of the vitamin when administered under different conditions. In the following pages an investigation of the influence of the sex of the animal, the level of intake, the source of the vitamin and the mode of administration on the storage of vitamin A in the liver of the rat is reported.

PROCEDURE

Weanling rats from our breeding colony were maintained in air conditioned quarters (75°F., 40% relative humidity) on the U.S.P. vitamin A-free diet composed of the following ingredients: purified casein, 18%; acetone-washed cornstarch, 65%; salt mixture, 4% (Jones and Foster, '42); acetone washed brewer's yeast, 8%; cottonseed oil, 5%; vitamin D, 6 units per gm of ration. When their weights remained stationary or declined for a period of 7 days they were considered to be depleted; this required from 25 to 35 days. They were

then divided into comparable groups of 6 and treated for 3 days with daily supplements of vitamin A. The supplements were administered orally by pipette unless otherwise stated. A short term treatment period was adopted in accordance with the observations that this method had given results which were as accurate as long term experiments (Sherman and Todhunter, '34; Guggenheim and Koch, '44).

In these studies on liver storage the daily level of vitamin A ranged from 250 to 1000 International Units (I.U.) per rat. This range was arbitrarily chosen since it had been found in preliminary work that the minimum doses required for normal growth did not promote any liver storage of the vitamin. To promote a rapid accumulation of vitamin A in the liver it was necessary to administer much more than the minimum required dose.

After the 3-day treatment the animals were rested for 1 day and on the fifth day were killed by decapitation. The livers from each group of rats were weighed, pooled, homogenized in a Waring blendor and samples taken for determination of vitamin A. In our early work determinations were made on individual livers but the small variations obtained did not justify this procedure.

Two to 5 gm samples of liver tissue were weighed and mixed with 60% KOH (0.5 ml/gm liver) and 95% alcohol (5.0 ml/gm liver). The mixture was heated on a steam bath until saponification was complete. The samples were then transferred to separatory funnels, water was added (10.0 ml/gm liver) and the tissue extracted twice with 50 ml portions of peroxide-free ethyl ether. Ethyl ether was used because Benham ('44) had reported that it gave more complete extraction of vitamin A than petroleum ether. The ether extract was washed thoroughly with distilled water until the wash water gave no color with phenolphthalein. After washing, the extract was dried by filtering through anhydrous Na_2SO_4, the ether removed by vacuum distillation and the residue dissolved in chloroform. The vitamin A content of the solution was determined by the Carr-Price reaction (Carr

and Price, '26) using the Evelyn colorimeter with filter no. 620. One ml of the chloroform solution was dispensed into a standardized colorimeter tube, the tube placed in the colorimeter and 9 ml of a 22% solution of antimony trichloride in chloroform were added rapidly. A reading was taken at the first momentary stop of the galvanometer needle and the "L" value, or optical density, corresponding to the galvanometer reading was calculated ($L = 2 - \log G$). This result was then converted to International Units of vitamin A using a reference curve. A vitamin A distillate (no. 78794),[1] which contained 401,000 I.U. per gm was employed as a standard in preparing the above curve.

Both the preparations used as supplements and the liver tissues were assayed by the Carr-Price method and the vitamin A content of each evaluated from the standard reference curve. All determinations were made in duplicate and repeated if the deviation of duplicate values exceeded 3%.

RESULTS

Relation between the sex of the animal and liver storage of vitamin A

Using the method described in the preceding section depleted male and female animals were treated with 500 units per day of the vitamin A distillate. This experiment was repeated 4 times at different intervals. Using the per cent of vitamin A stored as the criterion, no difference was found between the males and females; males stored 32.5% whereas 31.5% of the ingested vitamin was found in the female livers (table 1).

In 1942 Brenner et al. reported studies concerning the relation of sex to vitamin A storage in the white rat. Their animals were fed 14,000 units of vitamin A daily for 35 days and then placed on a vitamin A-free diet. The liver stores of vitamin A were determined at the onset of the depletion period and at various intervals thereafter. The initial determina-

[1] Obtained from Distillation Products, Rochester, N. Y.

tion showed that the livers of females contained slightly greater amounts of vitamin A. After 1 week on the A-free diet the liver stores of males were only two-thirds those of the females when the results were computed as units of vitamin A per 100 gm of body weight. When the calculations were made on the basis of vitamin content of the total livers the difference was not as marked because males have larger livers. Brenner and associates concluded from these data that females have a greater capacity for storage of vitamin A.

TABLE 1

Relation between sex of animal and liver storage of vitamin A.

NO. RATS	SEX OF RATS	INTAKE OF VITAMIN A DISTILLATE NO. 78794	LIVER STORAGE OF VITAMIN A		STORAGE
		I.U./day	*I.U./liver*		*%*
6	Male	500	417		27.8
4	Male	500	434		28.9
6	Male	500	567		37.8
6	Male	500	530		35.3
				Average	32.5
6	Female	500	364		24.3
8	Female	500	500		33.3
6	Female	500	520		34.7
6	Female	500	507		33.8
				Average	31.5

The apparent discrepancy between Brenner's work and the foregoing experiment might be explained by the longer preliminary treatment period which he used in contrast with the 3-day treatment employed here.

Relation between intake of vitamin A distillate no. 78794 and storage in the liver of the rat

The following study was undertaken to determine the relative amounts of vitamin A stored in the liver as the intake was increased. Daily doses ranging from 63 to 118,400 I.U.

per rat were administered to depleted animals and the treatment continued for 3 days. The vitamin A distillate, used as a standard throughout this work, was the source of vitamin A.

The data in table 2 show that at the lowest dosage, 63 units per day, the per cent of the vitamin that was stored was relatively small (11.1%); but the values increased with larger doses until a maximum was reached at a level of 2,000 to

TABLE 2

Relation between intake of vitamin A distillate and storage in the liver of the rat.

NO. OF RATS	INTAKE OF VITAMIN A DISTILLATE NO. 78794	LIVER STORAGE OF VITAMIN A	STORAGE
	I.U./day	*I.U./liver*	%
5	63	21	11.1
6	125	92	24.5
6	250	215	28.6
6	500	480	32.0
18	1,000	1,032	34.4
6	2,000	2,220	37.0
6	4,000	4,530	37.8
5	8,000	8,000	33.3
6	16,000	13,900	29.0
5	32,000	21,200	22.1
5	48,000	25,400	17.6
6	64,000	29,950	15.6
6	80,000	31,800	13.3
6	118,400	49,200	13.9

4,000 units per day (37%). From this point on, although an increase in intake produced an increase in liver vitamin A, the percentage stored decreased until at 118,400 units per day only 13.9% of the dose administered was found in the liver. Thus, it is apparent that raising the daily intake above the optimum storage level did result in an increase in the amount of the vitamin in the liver but this was accompanied by a decrease in the percentage of the original dose that was stored.

Relation between the intake of vitamin A from various sources and storage in the liver of the rat

Samples of vitamin A from various sources were chosen in order to compare their relative storage efficiency in the liver. The oils tested included vitamin A distillate no. 78794 which has been discussed in a previous section; vitamin A acetate; two types of fish liver oils, halibut and cod; and a saponified vitamin A concentrate. The concentrate was a fish liver oil which had been saponified in order to separate the vitamin A from impurities present in the crude oil. The unsaponifiable fraction which contained vitamin A in the alcohol form was extracted with ether and then concentrated by evaporating the solvent.

Vitamin A from each of these sources was fed at 3 levels: 250, 500 and 1,000 units per day, and the percentage stored in the liver was determined. The summarized results in table 3 indicate that with the exception of some samples of cod liver oil, vitamin A was stored equally well regardless of the source. Cod liver oil no. 92995 produced slightly lower liver stores of vitamin A than did the other oils administered. It has been reported (Oser et al., '43; '45) that cod liver oils are relatively unstable and tend to oxidize, which might possibly explain the lowered liver storage values reported here. Oser et al. had noted changes in the shape of the ultra-violet absorption curve during the oxidation of vitamin A and found that an upswing of the left leg of the curve with a lower extinction coefficient at the absorption maximum of 328 mμ was typical of oxidized vitamin A. In order to check cod liver oil no. 92995 for evidence of similar deterioration an ultraviolet absorption curve was obtained but the shape was characteristic of vitamin A with a peak at 327 mμ, which seemed to indicate that no oxidative change had occurred. Four additional samples of cod liver oil were then selected and used for feeding experiments. Two of these (no. 93445, no. 8955) produced somewhat lower liver stores while the vita-

min A from cod liver oils no. 7065 and no. 8945 was stored as well as that from other sources investigated. All cod liver oils were assayed by colorimetric, biological and spectrophotometric methods to insure an accurate estimate of the potency. Good agreement was noted between the values obtained by the 3 methods indicating that accurate measurements of the vitamin A administered had been made.

TABLE 3

Relation between intake of vitamin A from various sources and storage in the liver of the rat.

NO. OF RATS	VITAMIN A INTAKE	VITAMIN A IN LIVER	STORAGE	NO. OF RATS	VITAMIN A INTAKE	VITAMIN A IN LIVER	STORAGE
	I.U./day	*I.U./liver*	%		*I.U./day*	*I.U./liver*	%
	Vit. A distillate no. 78794				Cod liver oil no. 93445		
6	250	215	28.6	5	250	83	11.1
6	500	480	32.0	6	500	358	23.9
18	1000	1032	34.4	6	1000	755	25.2
	Halibut liver oil no. 92295				Cod liver oil no. 7065		
6	250	238	31.7	6	250	214	28.5
6	500	487	32.5	3	500	550	36.7
6	1000	1060	35.3	5	1000	835	27.8
	Vit. A concentrate no. 5822				Cod liver oil no. 8945		
6	250	233	31.0	6	250	195	26.0
6	500	437	29.1	6	500	454	30.3
5	1000	962	32.1	6	1000	1008	33.6
	Vit. A acetate no. 94265				Cod liver oil no. 8955		
5	250	185	24.7	6	250	138	18.4
6	500	480	32.0	5	500	348	23.2
5	1000	890	29.7	6	1000	630	21.0
	Cod liver oil no. 92995						
6	250	155	20.7				
11	500	269	17.9				
12	1000	625	20.8				

It was, therefore, concluded that with the exception of some samples of cod liver oil which gave sub-optimal liver storage, vitamin A was equally utilized and stored whether fed as a distillate, acetate, cod liver oil, halibut liver oil, or as a saponified concentrate.

In 1940 Gray and associates published the results of somewhat similar experiments in which equal amounts of vitamin A as U.S.P. Reference Oil, vitamin A caproate, distilled ester concentrate, vitamin A stearate, vitamin A alcohol and β-carotene were administered. Their data showed that slightly lower liver storage resulted from treatment with the alcohol form of the vitamin but when the experimental error was taken into consideration the significance of this difference seemed questionable. Animals receiving β-carotene were found to have very low vitamin A reserves but the remaining preparations produced equal liver stores.

Storage of vitamin A in rat liver following oral, subcutaneous, or intramuscular administration

Vitamin A distillate no. 78794 at a level of 500 units per day was administered to depleted rats orally or injected, either subcutaneously or intra-muscularly, as shown in table 4. The oil preparation was diluted in corn oil so that the animals received 0.1 ml per day for the 3-day period. The same dilution was used for all modes of treatment. As in the previous experiments, no treatment was given on the fourth day and

TABLE 4

Storage of vitamin A in the liver of the rat following oral, subcutaneous, and intramuscular administration of vitamin A distillate no. 78794.

NO. RATS	LEVEL OF VITAMIN A GIVEN	MODE OF ADMINISTRATION	LIVER STORAGE OF VITAMIN A	STORAGE
	I.U./day		*I.U./liver*	*%*
6	500	Oral	543	36.0
5	500	Oral	525	35.0
			Average	35.5
6	500	Subcutaneous	150	10.0
4	500	Subcutaneous	217	14.5
			Average	12.3
4	500	Intramuscular	11	0.7
5	500	Intramuscular	6	0.4
			Average	0.6

the animals were killed by decapitation on the fifth day preliminary to the determination of vitamin A in the livers. Liver stores of vitamin A were believed to indicate the amount of the vitamin which had been absorbed by rats which were treated for 3 days and killed at the beginning of the fifth day.

Oral administration of the vitamin produced optimum liver storage; subcutaneous treatment allowed less storage and the intramuscular method was the least effective. Vitamin A administered subcutaneously was approximately 35% as effective as the same amount given orally; intramuscular injection was about 2% as efficient as oral treatment. A relatively short time was allowed for the absorption of injected vitamin A when the 3-day treating technique was used and it might be assumed that under these conditions the vitamin A would not be completely utilized. It can be seen, however, from the growth studies outlined below that daily injections of vitamin A continued for 4 weeks did not afford maximum utilization. An inferior response was observed when parenteral administration of vitamin A was employed irrespective of the time allowed for absorption of the injected oil.

Growth response to oral, subcutaneous, and intramuscular administration of vitamin A

Vitamin A-depleted rats were treated daily for 4 weeks with vitamin A distillate no. 78794 given orally, subcutaneously, or intramuscularly at 2 different levels (table 5) and the utilization of vitamin A was indicated by the increase in growth. The growth response data paralleled the liver storage data in that they showed oral administration to be superior to parenteral treatment.

In agreement with the present work it has been reported that vitamin A oils are more effective orally than when injected, both in supporting normal growth and promoting liver storage (Koehne and Mendel, '29; Greaves and Schmitt, '37; With, '39; Barlow and Kocher, '42; Lease et al., '42). Although most investigators have used lower levels of vitamin A

and continued the treatment for various periods ranging from 4 to 6 weeks, their results are in accord with those reported here.

The close correlation between weight response and liver storage in depleted rats following administration of vitamin A indicates that the measurement of liver reserves following a 3-day treatment period is a reliable method for judging the effectiveness of a treatment.

TABLE 5

Growth response following oral, subcutaneous and intramuscular administration of vitamin A distillate no. 78794.

GROUP	LEVEL VITAMIN A GIVEN	METHOD OF ADMINISTRATION	NUMBER OF RATS Started	NUMBER OF RATS Finished	AVERAGE WEIGHT GAIN (28 DAYS)
	I.U./day				*gm*
1	2.04	Oral	20	19	30
2	4.08	Oral	20	17	60
3	2.04	Subcutaneous	20	2	—9
4	4.08	Subcutaneous	20	4	3
5	2.04	Intramuscular	20	1	—21
6	4.08	Intramuscular	19	4	—7

SUMMARY

1. Equal liver stores of the vitamin were found in both male and female animals when depleted rats were treated for 3 days with large amounts of vitamin A.

2. The relative amount of vitamin A stored in the liver was determined after treatment with increasing doses. At the lowest intake of 63 units per day the liver storage was 11% of the total dose while maximum storage (37%) was obtained at a daily level of 2,000 to 4,000 I.U. Further increase in the amount of vitamin A administered was accompanied by a steady decrease in the efficiency of storage.

3. A comparison was made of the liver storage resulting from treatment with different types of vitamin A preparations. It was found that different lots of cod liver oil, although administered at equal unit levels, produced variable liver reserves of vitamin A. With the exception of some lots of cod

liver oil which gave sub-optimal storage, vitamin A was utilized and stored equally well whether fed as a distillate, acetate, halibut liver oil, or saponified concentrate.

4. The storage of vitamin A in the livers of rats following oral, subcutaneous, or intramuscular administration was investigated. The oral method was found to be most effective in producing liver storage. Vitamin A injected subcutaneously was approximately 35% as efficient over a 5-day period as the same amount given orally, while intramuscular injection was only 2% as effective as the oral route.

5. Depleted rats were treated daily for a period of 4 weeks with vitamin A given orally, subcutaneously, and intramuscularly and the increase in growth was measured. It was found that vitamin A distillate was more effective orally than parenterally in promoting growth as well as in producing liver stores.

LITERATURE CITED

BARLOW, O. W., AND H. KOCHER 1942 Utilization by the rat of vitamin A administered perorally and intramuscularly. Am. J. Physiol., *137:* 213.

BAUMANN, C. A., B. M. RIISING AND H. STEENBOCK 1934 Fat-soluble vitamins. XLII. The absorption and storage of vitamin A in the rat. J. Biol. Chem., *107:* 705.

BENHAM, G. H. 1944 The antimony trichloride method for the determination of vitamin A. Canad. J. Res., *22:* 21.

BRENNER, S., M. C. HESSLER BROOKES AND L. J. ROBERTS 1942 The relation of liver stores to the occurrence of early signs of vitamin A deficiency in the white rat. J. Nutrition, *23:* 459.

CARR, F. H., AND E. A. PRICE 1926 Color reactions attributed to vitamin A. Biochem. J., *20:* 497.

GRAY, E. LE. B., K. C. D. HICKMAN AND E. F. BROWN 1940 The state of vitamin A in the liver of the rat after feeding various forms of the vitamin. J. Nutrition, *19:* 39.

GREAVES, J. D., AND C. L. A. SCHMIDT 1937 Relation of certain bile acids to absorption of β-carotene in the rat. Proc. Soc. Exp. Biol. Med., *36:* 434.

GUGGENHEIM, K., AND W. KOCH 1944 A liver storage test for the assessment of vitamin A. Biochem. J., *38:* 256.

JONES, J. H., AND C. FOSTER 1942 A salt mixture for use with basal diets either low or high in phosphorus. J. Nutrition, *24:* 245.

KOEHNE, M., AND L. B. MENDEL 1929 The utilization of fatty oils given parenterally. J. Nutrition, *1:* 399.

LEASE, J. G., E. J. LEASE, H. STEENBOCK AND C. A. BAUMANN 1942 The comparative physiologic value of injected carotene and vitamin A. J. Lab. and Clin. Med., *27:* 502.

MCCOORD, A. B., AND E. M. LUCE-CLAUSEN 1934 The storage of vitamin A in the liver of the rat. J. Nutrition, *7:* 557.

MOORE, T. 1931 Vitamin A and carotene. VII. The distribution of vitamin A and carotene in the body of the rat. Biochem. J., *25:* 275.

OSBORNE, T. B., AND L. B. MENDEL 1918 Nutritive factors in animal tissues. J. Biol. Chem., *34:* 17.

OSER, B. L., D. MELNICK AND M. PADER 1943 Chemical and physical determinations of vitamin A in fish liver oil. Ind. Eng. Chem., Anal. Ed., *15:* 717.

OSER, B. L., D. MELNICK, M. PADER, R. ROTH AND M. OSER 1945 Determination of vitamin A — correlation of improved spectrophotometric and colorimetric methods with multiple-level bioassays. Ind. Eng. Chem., Anal. Ed., *17:* 559.

SHERMAN, H. C., AND L. C. BOYNTON 1925 Quantitative experiments upon the occurrence and distribution of vitamin A in the body, and the influence of the food. J. Am. Chem. Soc., *47:* 1646.

SHERMAN, H. C., AND E. N. TODHUNTER 1934 The determination of vitamin A values by a method of single feedings. J. Nutrition, *8:* 347.

WITH, T. K. 1939 The effect of vitamin A given parenterally. Nord. Med., *3:* 2906.

STUDIES IN AMINO ACID UTILIZATION

I. THE DIETARY UTILIZATION OF MIXTURES OF PURIFIED AMINO ACIDS IN PROTEIN-DEPLETED ADULT ALBINO RATS [1]

L. E. FRAZIER, R. W. WISSLER, C. H. STEFFEE,
R. L. WOOLRIDGE AND P. R. CANNON
Department of Pathology, University of Chicago, Illinois

FOUR FIGURES

(Received for publication August 20, 1946)

The substitution of mixtures of purified amino acids for dietary protein in rations adequate in calories, vitamins and minerals has helped greatly to clarify understanding of the nutritive potentialities of proteins. It is now possible to prepare a mixture of the 10 so-called essential amino acids which promotes growth in young rats (Kinsey and Grant, '44), or mice (Bauer and Berg, '43), or maintains nitrogen balance in dogs (Rose and Rice, '39) and adult man (Rose, Haines, Johnson and Warner, '43). Many questions remain unanswered, however, concerning the mechanisms governing the utilization of these amino acids. The question of the indispensability of arginine and histidine needs further study.

[1] The subject matter of this paper has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the armed forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

The work has been aided, also, by the John and Mary R. Markle Foundation, The National Livestock and Meat Board, The Douglas Smith Foundation for Medical Research of the University of Chicago, and the Allen B. Wrisley Company.

More information is needed regarding such problems as the possible toxicity of some of the unnatural forms (Albanese and Irby, '43), the optimal amounts of each amino acid in the daily ration, the role of accessory substances such as strepogenin (Woolley, '45) in the synthesis of proteins from amino acid mixtures, and the possible adverse effects from the use of improperly-balanced mixtures even though all of the indispensable amino acids may be present.

In this and the following paper all observations were made on a group of protein-depleted rats fed rations whose nitrogen source, other than traces in the cornstarch and vitamins, consisted of a mixture of either 16 (ration A), 10 (ration B), or 9 (ration C), purified amino acids. In these 2 papers we present evidence concerning the influence of these rations upon (a) weight-recovery; (b) food consumption; (c) plasma and red cell volumes and regeneration of serum proteins and erythrocytes. Although the data are from experiments dealing with only 28 rats, they are presented as illustrative examples. As a matter of fact, the experiments have been confirmed with 55 additional rats so that, in reality, they represent findings from the use of more than 85 different animals.

The "sixteen amino acid mixture" utilized was patterned after the amino acid composition of casein (Block and Bolling, '45), except that serine, proline, and hydroxyproline were absent and the racemic mixtures of threonine, valine and isoleucine were used with the assumption that the unnatural *d* forms do not replace their respective enantiomorphs metabolically. Although the carbon chains of these *d* amino acids may not be used for the synthesis of tissue protein, their amino nitrogen may be utilizable for the synthesis of non-essential amino acids. Accordingly, an amount of glutamic acid equivalent to the non-utilizable *d* forms was omitted from the mixture. Correction was made for HCl in the arginine, histidine and lysine samples and for water of crystallization in the latter two. Whenever an amino acid was omitted from the mixture in order to observe the effect of its deficiency, its

equivalent in moles of glutamic acid was substituted. When amino acid mixtures were made containing only 10 or 9 essential amino acids, the ratios of the amino acids were held constant while their concentrations were increased in order to keep the dietary nitrogen levels the same. The amounts of

TABLE 1

Composition of amino acid mixtures employed to supplement basal ration 4 E.

		Percentage composition of mixtures of amino acids in			Mg of essential (l) amino acids per 15 gm diet per rat day in		
		Ration A	Ration B	Ration C	Ration A	Ration B	Ration C
l (+)	Arginine HCl	4.214	6.592	.....	63.4	99.1	....
l (+)	Histidine HCI	2.871	4.491	4.808	38.5	60.4	64.7
dl	Isoleucine	11.050	17.286	18.505	100.0	157.2	168.3
l (+)	Leucine	10.285	16.186	17.224	190.0	294.3	313.1
l (+)	Lysine HCl	8.051	12.594	13.483	106.0	167.2	178.5
dl	Methionine	2.975	4.654	4.982	54.0	84.6	90.6
dl	Phenylalanine	4.420	6.914	7.402	80.0	125.7	134.6
dl	Threonine	6.630	10.371	11.103	60.0	94.3	100.9
dl	Tryptophane	1.530	2.393	2.562	27.7	43.5	46.6
dl	Valine	11.900	18.615	19.929	108.0	169.2	181.2
dl	Alanine	4.760					
dl	Aspartic acid	5.355					
l (—)	Cystine	0.306					
l (+)	Glutamic acid	20.157					
	Glycine	0.425					
l (—)	Tyrosine	5.440					
		100.069	99.999	99.998			

each amino acid present in the different mixtures are given in table 1.[2] In the graphs, the ration containing 16 amino acids was originally referred to as AAR. For greater convenience it is referred to in these papers as ration A. Similarly, the ration containing the 10 "essential" amino acids, referred to

[2] Most of the amino acids were purchased from Merck and Co. Alanine, glycine, isoleucine, threonine, tyrosine and valine were purchased from General Biochemicals, Inc. Some methionine was purchased from the Pfanstiehl Co. Purity of the amino acids was checked by Kjeldahl determinations in the case of the racemic forms and by optical rotation determinations in the case of the active natural forms.

in the graphs as EAAR10 or EAAR, is here called ration B; and the 1 containing only 9 essential amino acids, shown in the graphs as 9AA or 9EAAR, is here called ration C.

EXPERIMENTAL PROCEDURES

Male, albino rats of the Sprague-Dawley strain, with initial weights varying from 220 to 244 gm, were subjected to protein depletion according to procedures previously described (Wissler, Woolridge, Steffee and Cannon, '46). Selection was made from groups of rats which had been on a low protein "depletion" diet (3E) for approximately 3 months, animals being chosen on the basis of the uniformity in initial weights, percentages of weight loss (25–33%) and concentrations of serum proteins (4.06–4.85%) and hemoglobin (11.2–15.3%). The rats were placed in individual cages prior to the incorporation of the amino acid mixtures or casein into the basal ration for use in the "repletion" period. All rations were kept isocaloric. Two days before the experiments were to be started the animals were weighed, and bled (tail vein) in order to determine the serum protein and hemoglobin concentrations. On the basis of these data as well as the initial predepletion weights and percentage weight losses, the most comparable animals were paired, 1 animal of each pair being offered a weighed amount (15 gm per rat per day) of ration A, while its mate was fed this ration with one "essential" amino acid omitted. Water was offered ad libitum. For comparison, other pairs of rats were fed the 4E basal or casein rations under similar circumstances and concentrations of nitrogen (table 2). Blood volumes were determined on the first day of the experiment. During the first dietary period the amounts of ration eaten, wasted or rejected were determined daily and the animals were weighed frequently. On the tenth day the rats were fed in the morning but were fasted for the last 12 hours of the 24-hour period. On the morning of the eleventh day, weights, serum protein and hemoglobin concentrations and blood volumes were determined. In most instances thereafter the diets of each pair of animals were reversed for a

second 10-day period during which weights and diet-consumption figures were determined as before. At the end of the 20-day period the animals were again fasted for 12 hours and the various observations repeated after which they were sacrificed and autopsied.

TABLE 2

Composition of rations used.

INGREDIENTS PER 100 GM	4 E BASAL	CASEIN	AMINO ACIDS
Nitrogen source	0.0	10.46	12.12
Corn oil	4.0	4.0	4.0
Cornstarch	71.0	61.7	58.88
Ruffex	5.0	5.0	5.0
Salt mixture [1]	4.0	4.0	4.0
Vitamin solutions	2.7	2.7	2.7
Water	13.3	12.14	13.33
VITAMINS PER 100 GM OF RATION			
Choline chloride	0.20 gm		
Oleum percomorphum	0.30 drops		
Nicotinamide	2.84 mg		
Pantothenate Ca	1.75 mg		
Pyridoxine HCl	0.64 mg		
Riboflavin	1.08 mg		
Thiamine HCl	0.54 mg		

[1] Hawk and Oser's ('46) modification of the Osborne and Mendel salt mixture plus 1 gm each of copper sulfate, and zinc chloride added to the trace elements.

The utilizable amounts of each of the essential amino acids supplied per 15 gm of ration for the "amino acid rations" are given in table 1. From these data it is possible to compute the milligrams of amino acids eaten per rat day, knowing the actual amount of food consumed.

EXPERIMENTAL FINDINGS

The effects of ration A upon weight-recovery are shown graphically in figure 1, where the starting weights have been connected with the 10-day and 20-day values by straight lines. It will be noted in every instance that the animal which ate this ration for 10 days gained weight rapidly, an average of

between 4 and 5 gm per day, whereas each animal which ate the ration with any one of 9 indispensable amino acids omitted continued to lose weight. Furthermore, comparison of these results with those of a sample pair of the animals which ate the 4E basal ration alone or this ration supplemented with casein (fig. 2) shows a less rapid weight recovery for the rat which ate the natural protein than for several of those consuming ration A. Moreover, the animals which ate the 4E ration alone lost less weight, in general, than did the animals which ate the ration containing fifteen amino acids but with 1 indispensable amino acid omitted. In other words, the animals did slightly better on no protein at all than on a mixture of 15 amino acids but with 1 indispensable amino acid lacking. Exception occurred only with respect to lysine deficiency, where weight loss was practically identical with that for the rats fed the 4E ration. With respect to arginine, its presence or absence seemed to make but little difference. One rat, in fact, kept on an arginine deficient diet for 30 days, recovered almost as much lost weight as did its companion which ate ration A; furthermore, spermatogenesis as judged by histological study was as well reestablished in the arginine-deficient rat as in the other. It is obvious, therefore, that the protein-deficient rats were able to synthesize arginine with sufficient rapidity almost to keep pace with those supplied with arginine.

In view of the unsuccessful use by Albanese and Irby ('43) of a mixture of 10 amino acids for growth of young rats, and because of their suggestion of the possibility of toxic *d* forms being present in such a mixture, we prepared a mixture of 10 amino acids (table 1) and incorporated it in the basal ration at the same nitrogen concentration employed for ration A. Figure 2 demonstrates the results obtained from the feeding of these 2 types of rations. It is obvious that weight recovery was as good with only 10 amino acids in the ration as with the 16 of ration A. It is evident, also, that there was no apparent "toxic" effect from the use of a mixture containing several of the unnatural *d* forms.

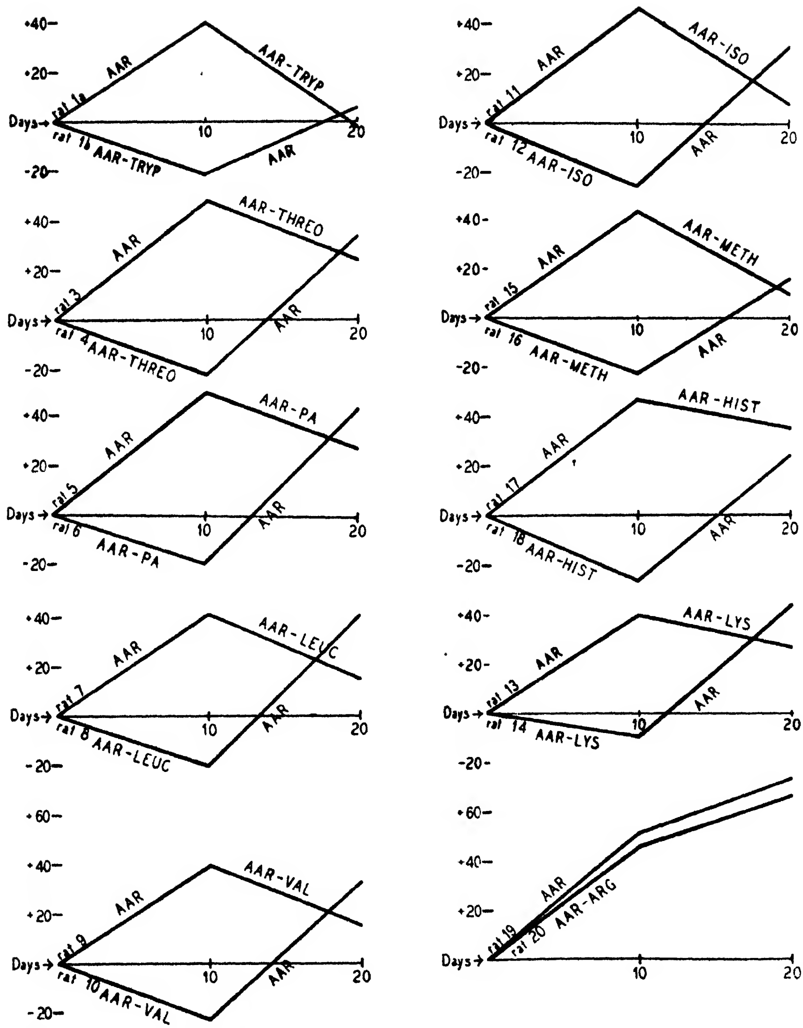


Fig. 1 Comparison of weight changes in protein-depleted rats fed ration A (AAR) and ration A devoid of 1 indispensable amino acid. After 10 days the rations were reversed. Note the common pattern except in the case of arginine.

Although it is apparent that a protein-deficient rat can recover lost weight when only 10 amino acids are present in the ration, we wished to secure additional evidence regarding the dispensability of arginine. Accordingly, a mixture of amino acids containing only 9 indispensable amino acids was prepared (table 1, ration C), but, again, with as much total nitrogen as was present in rations B and A. This mixture when added to the basal ration, at a comparable concentration of nitrogen, yielded essentially as good a weight recovery rate as did ration A, fed simultaneously (fig. 2).

One of the most striking features of the experiments in which the rats were offered rations deficient in any one of the 9 indispensable amino acids, was their disinclination to consume the ration. In other words, these 9 amino acids are essential, also, for good appetite, and omission from the ration of any 1 of them, even though its nitrogen equivalent is replaced by glutamic acid, is followed quickly by reduced food-consumption. On the other hand, addition to the ration of the missing amino acid, is followed just as promptly by improved appetite. The data, which record the amounts of food consumed, are shown graphically in figures 3 and 4 in which the rectangles are designated "food-consumption areas." The 4 rectangles for each amino acid tested represent the proportions of ration (15 gm per rat per day) eaten daily by the protein-depleted rats for each 10-day period. The upper left-hand rectangle represents the amount of food eaten by the rat fed ration A, the lower left hand one, the amount of food eaten after 1 amino acid had been omitted from the ration.

Fig. 2 (Upper left). Comparison of weight changes in 2 rats, one of which was fed ration A (AAR) and the other ration B (EAAR10), both for 20 days. (Upper right). Weight changes in 2 rats one of which was fed ration A for 20 days (no. 211–4) and the other (no. 214–1) one fed this ration with the lysine injected subcutaneously daily for 10 days and then replaced by salt solution. (Lower left). Weight changes in rats fed casein ration and protein-free ration for 10 days and after reversal of rations. (Lower right). Comparison of weight changes in 2 rats fed ration A and ration C (9AA) for 20 days. All rats were protein-depleted at the beginning of the experiments.

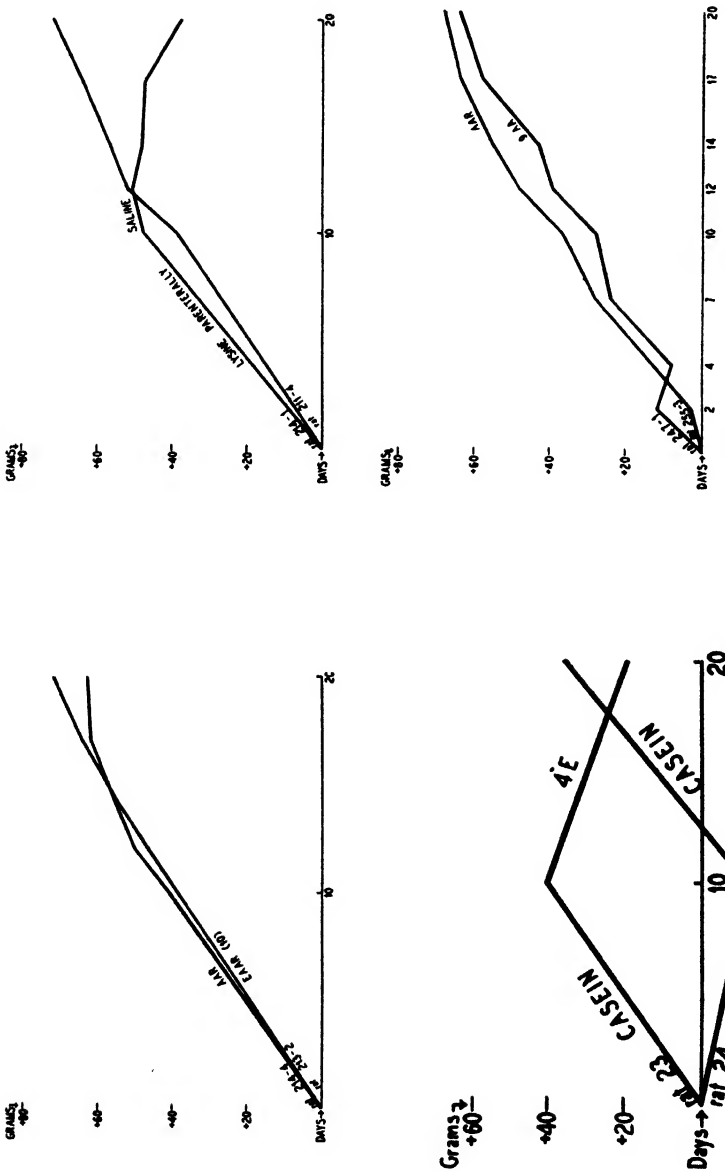

SALINE
LYSINE PARENTERALLY
AAR
EAAR (10)
9 AA
CASEIN
4E
CASEIN
4E
Grams
Days

Figure 2

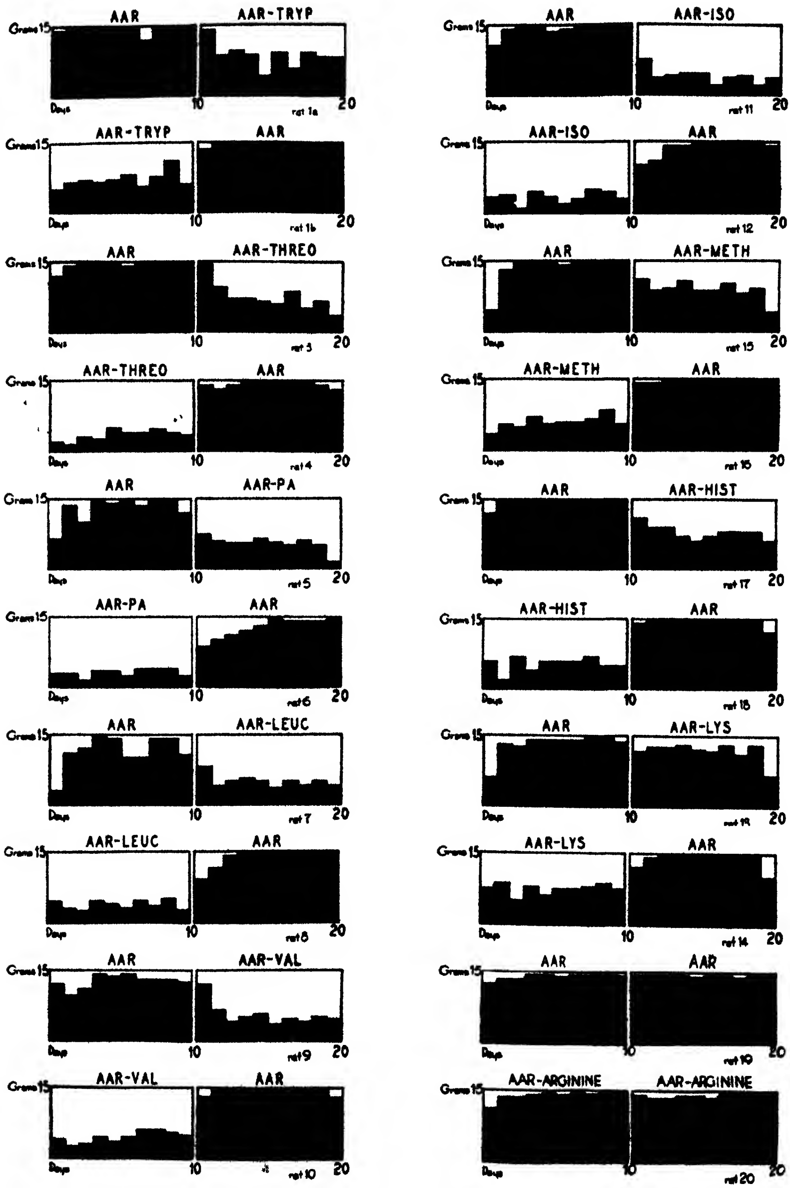


Fig. 3 "Food consumption areas" resulting from the feeding of rations, to be examined in relation to figure 1. Note the common pattern except in the case of arginine.

The 2 rectangles on the right indicate amounts of food consumed by each rat for a second 10-day period after reversal of the rations. Inspection of the rectangles demonstrates in every instance that food-acceptance is correlated with the presence or absence of 1 of the 9 indispensable amino acids.

A more detailed examination of these "food consumption areas" will illustrate more definitely the effects observed. For example, rats 1a and 2b were offered rations identical except

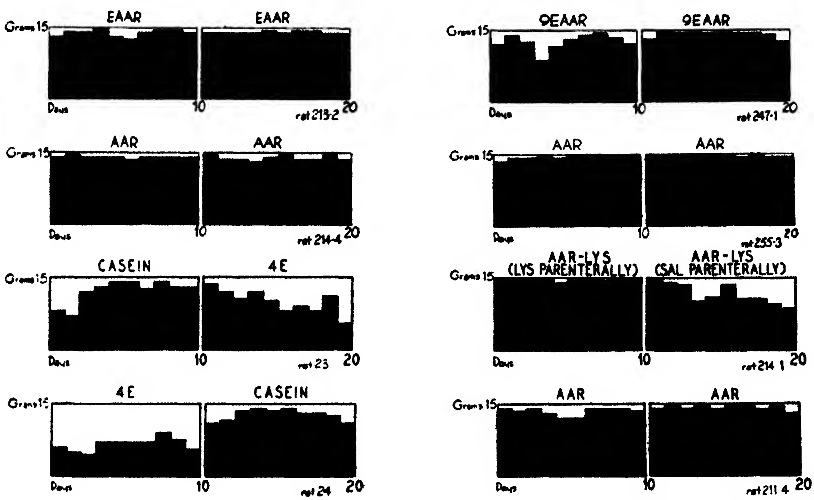


Fig. 4 "Food consumption areas" to be compared with weight changes of figure 2 in rats fed the various types of rations.

for the presence or absence of approximately 29 mg of dl-tryptophane per 15 gm of ration. As can be seen, rat 1a ate practically all of his daily ration for the first 10 days. During this time he recovered lost weight. On the eleventh day, although tryptophane was now absent from the ration, he ate 14 gm; but on the twelfth day he ate only 8.5 gm, and his daily food-consumption thereafter was always less than 10 gm. During this time he lost weight steadily. In contrast, rat 1b which was offered the ration devoid of tryptophane, ate poorly for the entire 10-day period and on only 1 day (the ninth) did he eat more than 10 gm. On the eleventh day, after tryptophane

had been added to the ration, he ate 13.5 gm, and on each of the succeeding 9 days he ate all of the ration; during this period he gained weight steadily. Rats 9 and 10 reacted similarly when valine was tested, After 10 days' consumption of ration A, rat 9 had made a good weight-recovery and had eaten the ration well. On the eleventh day, after approximately 216 mg of dl-valine had been removed from the ration, the rat ate the same amount of food which he had eaten on the preceding 4 days (12 gm per day). On the twelfth day, however, his food-consumption dropped to 6.5 gm and stayed low for the succeeding 8 days. In contrast, rat 10, when first offered the ration devoid of valine, refused for 10 days to eat more than 6 gm per day. On the eleventh day, after valine had been added to the ration, he ate 13 gm, and from then on he ate his daily ration almost completely. A similar pattern is seen, also, in the case of the 2 rats (numbers 3 and 4) offered rations with and without threonine.

Of interest, also, are the findings in the rats which responded to the addition of phenylalanine, leucine or isoleucine to the deficient rations. During the first 10 days these animals refused to eat well when offered rations devoid of any one of these amino acids. When, however, each of the missing amino acids was put into the rations, daily food-consumption improved in a step-wise fashion until practically maximal consumption was attained and maintained. These patterns suggest a gradual return of appetite, associated with a gradually increased ingestion of those amino acids in which the rations had been deficient. It is noteworthy, also, that this response occurred when such dl- forms as phenylalanine, valine, isoleucine and threonine were used, thus indicating the lack of any toxic effect from the unnatural forms supplied, as judged by appetite or weight recovery.

In an effort to ascertain whether these changes in appetite might depend upon taste, 2 animals were given rations differing only in the route by which lysine was administered, viz., 1 rat was fed ration A for 20 days while the second rat was fed this ration devoid of lysine but with the lysine injected sub-

cutaneously, 2 doses per day (146 mg 1 (+) lysine $HCl \cdot H_2O$ per rat day). As can be seen from figure 2, the rat which received lysine parenterally made an even better weight recovery than did the one which ingested lysine. At the end of 10 days, however, when salt solution was substituted for the injected lysine, the animal began quickly to lose weight, whereas the rat eating ration A continued steadily to gain weight. Evidence of this kind speaks against the idea that taste may be a determinant of appetite under these circumstances; on the other hand it does not eliminate the possibility that food consumption may be influenced by blood amino acid thresholds, at least until such a possibility can be studied more completely.

The degree of consumption of rations other than ration A is shown in figure 4. Examination of these "food-consumption areas" indicates that the protein-depleted rats maintained good appetites for 20 days when fed rations containing only 10 or 9 indispensable amino acids as the principal source of dietary nitrogen. Their food intake, in fact, equalled that of the animals eating the casein ration and exceeded that of the rats eating the basal ration. It is obvious, also, that when lysine was injected, the rat's appetite was as good as that of the animal eating the complete ration A but that appetite declined when salt solution was substituted for the injected lysine.

COMMENT

Although it has long been known that a loss of appetite may at times be an indicator of an amino acid deficiency, we have found no statements emphasizing the rapidity with which this may manifest itself. Rose ('38) has said that "growing animals lose the desire to eat when the food is not suitable for tissue-synthesis, but regain it when all of the components required for anabolism are made available" and that (Rose, '31–'32), "Our experience with other types of deficiencies involving the nitrogenous portion of the ration has taught us to expect a marked failure in appetite when the diet is completely devoid of an essential component . . . Hundreds of

experiments . . . involving the use of completely hydrolyzed proteins, have demonstrated that rats readily consume adequate amounts of diets in which the nitrogenous portion is in the form of mixtures of amino acids, provided all of the materials for growth are available." Smith ('31) has also remarked that "an experimental animal deprived of protein soon exhibits a complete loss of appetite."

Our findings corroborate these statements. They also demonstrate the surprising fact that food-acceptance by protein-depleted and presumably hungry rats may be determined by the presence or absence of only a few mg of a particular indispensable amino acid. For example, in experiments to be reported in more detail later, we found that a rat offered a ration containing approximately 18 mg of dl-tryptophane per 15 gm of ration ate almost all of the daily ration and gained 43 gm in 10 days. Another rat, however, offered the same ration containing only 9 mg of dl-tryptophane per 15 gm of ration had an average daily food-consumption for the 10-day period of 10 gm and gained but 4 gm of weight. In other words, the difference in weight recovery of the 2 animals was determined by the presence or absence in the daily ration of only 9 mg of dl-tryptophane. Similar effects have been observed with the other 8 indispensable amino acids although the quantitative requirements for each amino acid are different.

The mechanism governing this impairment of appetite in acute amino acid deficiency is of especial interest. Is it the result of a lowered taste-threshold due to the lack of certain amino acids, or is it due to a general metabolic disturbance whereby the sense of well-being of the animal is altered, as a result of which the rat becomes actually sick and hence is not interested in food? If the loss of appetite is due to inadequate stimulation of taste organs, it would appear necessary to assume the existence of 9 indispensable amino acid "essential," also, for taste and that omission of any one of them from the diet would depress the taste stimulus for all the others. Against the idea of taste, however, is the fact that in

several of our experiments the animal continued to eat well for 1 day after removal of the indispensable amino acid from the ration, after which food-consumption declined and remained low. If taste were the determining factor here it is difficult to see why it should require 24 hours for the rat to discover the absence of a particular amino acid from the ration and then to eat only one-half or less of his daily diet.

Evidence suggesting a general metabolic disturbance as the factor responsible for the impaired appetite is furnished by experiments (Rose et al., '42; '43) in which healthy young men were fed rations adequate in calories, vitamins and minerals but containing a mixture of 10 essential amino acids as the only source of dietary nitrogen. The men remained in nitrogen balance until any 1 of 8 amino acids was removed from the ration whereupon they went promptly into negative nitrogen balance. Readdition to the ration of the missing amino acid, however, brought them back quickly into nitrogen balance. Of especial interest is the fact that when the subjects were in negative nitrogen balance they felt miserable and had impaired appetites but that after nitrogen balance was reestablished they again felt better and their appetites improved.[3] It is possible, therefore, that a similar mechanism may operate in the rats and that their disinclination to eat may be due largely to the fact that they actually feel too "sick" to be interested in food, even though they are severely undernourished and need the food to promote effective convalescence.

The possibility should also be considered that the loss of weight and the associated impairment of appetite accompanying acute amino acid deficiency might be due, indirectly, to the restricted intake of calories, vitamins and minerals. In order to test this possibility, protein-depleted rats were gastrostomized and then fed through the gastrostomy tube. Although these experiments are still in progress, evidence obtained thus far indicates that an adequate intake of calories,

[3] Personal communication from W. C. Rose.

vitamins and minerals lessens but does not prevent the weight-loss of acute amino acid deficiency. For example, 1 rat fed the ration A via gastrostomy for 10 days gained weight steadily; but when tryptophane was removed from the ration he began to lose weight despite the fact that his caloric, vitamin and mineral intakes were unchanged. However, his weight loss was less than that of his control being offered the deficient ration ad libitum, thus suggesting that if acute amino acid deficiency induces some sort of "toxic" disturbance of internal metabolism, this is less severe in the presence of adequate calories, vitamins and minerals. At any rate, the mechanism responsible for the impaired appetite requires further study.

These consequences of acute amino acid deficiency emphasize further some of the difficulties which hamper efforts to evaluate quantitatively by biological methods the nutritive potentialities of proteins. If only a few mg of any 1 of 9 indispensable amino acids can determine either nutritive success or failure of a biological assay, it is not surprising that there should be so much controversy concerning the kinds of methods which should be utilized for determining protein quality. There is still no general agreement as to the nitrogen level optimal for protein-assay; and no biological method has as yet been developed which can determine the concentrations of each individual amino acid in a test-ration. Ultimately, it may become necessary to ascertain the limiting level of each indispensable amino acid in the protein being tested rather than to determine this indirectly as is now done. Further improvements in methods of amino acid analysis, both chemical and microbiological, may make this possible. Thus it may be possible to demonstrate absolute quantities of indispensable amino acids in a protein and their ratios one to another. Similarly, it may be possible to relate these values to those for the nonessential amino acids as well. However, even then, biological methods will still be needed because of the fact that, as a consequence of heat-injury or processing alteration, certain indispensable amino acids may be rendered metabolically

non-utilizable even though they are demonstrable by amino acid analysis.

It is of interest that these experiments failed to demonstrate the retarded weight gains observed by others (Woolley, '45; Rose and Womack, '46) in young animals fed rations whose nitrogenous constituents came from hydrolyzed protein or from mixtures of purified amino acids. Although we know of no source of strepogenin in the materials used in the rations of our experiments it should be mentioned that the ration 3E, used for the depletion period, contained 1% of Wilson's 20:1 liver concentrate. It is possible, therefore, that strepogenin from this source might have been stored in the depleted rats so that no deficiency was apparent in the relatively short "repletion" period. Cary et al. ('46) have presented evidence that an unidentified accessory growth factor present in liver, casein and some other foods, may be stored by young rats. Our rats are almost full-grown and may not need exogenous accessory factors as do young rats. At any rate, our experiments agree with those of Rose that, with the exception of arginine, the same 9 amino acids essential for adequate growth in the young rat are also essential for effective weight-recovery and good appetite in adult protein-depleted rats.

SUMMARY

Weight-recovery of comparable pairs of protein-depleted rats was determined while the animals were fed "amino acid rations" for a 10-day period. In most instances 1 rat was offered a ration containing a mixture of 16 amino acids patterned after the amino acid composition of casein while the second received the same mixture with one indispensable amino acid omitted. In most cases the rations were reversed for a second 10-day period. In some experiments fewer amino acids were employed. The results may be listed as follows:

1. Rats fed the amino acid ration containing 16 amino acids (ration A) gained weight rapidly, usually exhibiting as great or greater weight gains in 10 days as rats fed a ration con-

taining an isonitrogenous quantity of casein; they also ate the ration completely.

2. Omission, singly, of histidine, lysine, tryptophane, phenylalanine, methionine, threonine, leucine, isoleucine or valine from this ration led to marked loss of weight in a 10-day period, coincident with a prompt loss of appetite as evidenced by consumption of only from one-third to one-half of the daily ration offered. The weight loss in most cases was greater than that shown by comparable rats fed a low-protein basal ration for a similar period. When, however, the missing amino acid was added to the ration the rats quickly recovered lost appetite and rapidly regained lost weight.

3. When arginine was omitted from the ration the rats gained weight at rates approximately equal to those of similar rats receiving the complete amino acid ration and their appetites were well-maintained. No histological differences in spermatogenesis could be seen after 30 days' feeding of the respective rations.

4. A ration containing only the 10 amino acids indispensable for rat growth, fed at a nitrogen level equivalent to that in ration A, supported a weight gain equal to or greater than that of similar rats receiving the 16 amino acids. Similarly, when only the 9 amino acids listed above were fed, an equivalent rate of weight gain was obtained. With both types of ration good appetites were maintained over a 20-day period.

5. When 1 amino acid (lysine) was administered parenterally and the others were consumed by mouth, the weight increase was comparable to that of rats receiving the complete ration by mouth; good appetite was also maintained. When, however, salt solution was substituted for the lysine, both the animal's weight and appetite declined.

6. The rapidity with which appetite declines in these protein-depleted rats after omission of any one of 9 indispensable amino acids from the ration suggests the development of a state of acute amino acid deficiency which can be corrected only by the addition to the ration of adequate amounts of the missing amino acid. Furthermore, no evidence of a need for

an accessory polypeptide factor or factors to promote effective utilization of these amino acids was demonstrated by these experiments in which the only supplementary constituents were pure crystalline vitamins.

7. In the biological evaluation of protein, dilution of the test-protein to the point where acute amino acid deficiency occurs prevents an adequate quantitative estimation of nutritive quality. It is suggested that ultimately it may become necessary to evaluate proteins in terms of the limiting action of each indispensable amino acid in the test-protein rather than to attempt to evaluate amino acid levels indirectly by current methods.

LITERATURE CITED

ALBANESE, A. A., AND V. IRBY 1943 Observations on the biological value of a mixture of essential amino acids. Science, *98:* 286.

BAUER, C. D., AND C. P. BERG 1943 The amino acids required for growth in mice and the availability of their optical isomers. J. Nutrition, *26:* 51.

BLOCK, R. J., AND D. BOLLING 1945 The Amino Acid Composition of Proteins and Foods. C. C. Thomas, Springfield, Illinois.

CARY, C. A., A. M. GARTMAN, L. P. DRYDEN AND G. D. LIKELY 1946 An unidentified factor essential for rat growth. Fed. Proc. Am. Soc. Exp. Biol., *5:* 128.

HAWK, P. B., AND B. OSER 1946 Practical Physiological Chemistry. 12th ed. P. Blakiston and Co., Philadelphia.

KINSEY, V. E., AND W. M. GRANT 1944 Adequacy of the essential amino acids for the growth of the rat. Science, *99:* 303.

ROSE, W. C. 1931–32 Feeding experiments with mixtures of highly purified amino acids. I. The inadequacy of diets containing 19 amino acids. J. Biol. Chem., *94:* 155.

——— 1938 The nutritive significance of the amino acids. Physiol. Rev., *18:* 109.

ROSE, W. C., W. J. HAINES AND J. E. JOHNSON 1942 The role of the amino acids in human nutrition. J. Biol. Chem., *146:* 683.

ROSE, W. C., W. J. HAINES, J. E. JOHNSON AND D. R. WARNER 1943 Further experiments on the role of amino acids in human nutrition. J. Biol. Chem., *148:* 457.

ROSE, W. C., AND E. E. RICE 1939 The significance of the amino acids in canine nutrition. Science, *90:* 186.

ROSE, W. C., AND M. WOMACK 1946 Evidence for the existence of an unidentified growth stimulant in proteins. J. Biol. Chem., *162:* 735.

SMITH, A. H. 1931 Phenomena of retarded growth. J. Nutrition, *4:* 427.

WISSLER, R. W., R. L. WOOLRIDGE, C. H. STEFFEE AND P. R. CANNON 1946 The relationship of the protein reserves to antibody production. II. The influence of protein repletion upon the production of antibody in hypoproteinemic adult white rats. J. Immunol., *52:* 267.

WOOLLEY, D. W. 1945 Observations on the growth-stimulating action of certain proteins added to protein-free diets compounded with amino acids. J. Biol. Chem., *159:* 753.

STUDIES IN AMINO ACID UTILIZATION

II. ESSENTIAL AMINO ACIDS AS A SOURCE OF PLASMA PROTEIN AND ERYTHROCYTES IN THE HYPOPROTEINEMIC RAT [1]

E. P. BENDITT, E. M. HUMPHREYS, R. L. STRAUBE, R. W. WISSLER AND C. H. STEFFEE

Department of Pathology, University of Chicago, Illinois

(Received for publication August 20, 1946)

Because of the great interest in plasma protein production in acute and chronic states of hypoproteinemia, and in the correction of these conditions by means of orally and parenterally administered mixtures of amino acids, it is important to know which amino acids are necessary for the production of plasma proteins. In the preceding paper the effects of various combinations of amino acids and of single amino acid deficiencies on weight recovery and diet consumption of the protein-depleted rat have been described (Frazier et al., '46). In the present paper the influence of various amino acids on the fabrication of serum protein and of erythrocytes is described.

EXPERIMENTAL PROCEDURES

The present observations were made on the same animals used in the experiments described in paper I of this series. At the end of the protein-depletion period the rats were paired

[1] The subject matter of this paper has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

The work has been aided, also, by the John and Mary R. Markle Foundation, The National Livestock and Meat Board, The Douglas Smith Foundation for Medical Research of the University of Chicago, and the Allen B. Wrisley Company.

as described (Frazier et al., '46) and on the first day of the experimental period blood volume, serum protein and hematocrit determinations were made. The rats were then placed upon the test rations. At the end of 10 days these values were again determined, the diets of each pair of animals interchanged and a third set of values obtained after a second 10-day period.

Total circulating serum protein (T.C.S.P.) and total circulating erythrocyte volumes (T.C.E.V.) were computed from the determined values of total blood volume, plasma volume, serum protein and hematocrit. The determination of the blood volume using Evans Blue dye has been described in detail elsewhere (Benditt, Straube and Humphreys, '46). The method as used in the present experiments differs only slightly from that described. The points of difference are (a) in the dilution of the blood used for estimation of the dye concentration, and (b) the protein used in the diluting fluid. In the present investigations 0.2 ml of oxalated whole blood was diluted with 5.0 ml of diluting fluid which contained 2.0 gm of whole dehydrated beef plasma instead of the 2.0 gm of purified beef albumin described elsewhere. Serum proteins were determined by the method of Barbour and Hamilton ('26). Hematocrits were determined in capillary tubes as described.

From the measured concentration of dye in 0.2 ml of blood the total blood volume was calculated, and from this and the hematocrit value the total circulating red cells and the plasma volume were estimated. From the plasma volume and serum protein concentration the total circulating serum proteins were calculated.

RESULTS

In table 1 are presented the mean values and their standard errors for the quantities of plasma volume, serum protein concentrations, total circulating serum protein (T.C.S.P.), total circulating red blood cell volume (T. C. E. V.) and the body weight of a group of 8 animals fed the protein-free basal ration (4E) for 10 days, and another group of 7 animals fed the 9% casein ration (Cas) for 10 days. These data show a rea-

sonable constancy and compare favorably with other published figures on the same quantities measured in the rat (Metcoff and Favour, '44).

Table 2 presents the total circulating serum protein changes for the animals on the various diets. Column 1 contains the

TABLE 1

Averaged data for rats fed basal 4E and 9% casein rations.

	PLASMA VOLUME		SERUM PROTEIN		TOTAL CIRCULATING SERUM PROTEIN		TOTAL CIRCULATING RED CELL VOLUME		BODY WEIGHT	
	Day 0	Day 10	Day 0	Day 10	Day 0	Day 10	Day 0	Day 10	Day 0	Day 10
	ml		*gm %*		*mg*		*ml*		*gm*	
	Protein free (4E) diet (8 animals)									
Mean	6.31	5.90	4.08	4.40	258	257	3.81	3.73	160	145
S.E.	± .72	± .51	± .59	± .37	± 14	± 13	± .52	± .66	± 2	± 2
	9% casein (Cas) diet (7 animals)									
Mean	6.44	7.64	4.20	5.81	271	447	3.67	5.71	159	197
S.E.	± .66	± .71	± .66	± .44	± 15	± 19	± .66	± .65	± 2	± 2

TABLE 2

Changes in total circulating serum protein.

RATION	4E	CAS	RATION A	RATION A MINUS	RATION A MINUS	RATION A	
PERIOD (day)	0–10	0–10	0–10	10–20	0–10	10–20	
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*The missing amino acid*
	— 11	212	210	— 73	27	159	Tryptophane
	— 36	186			— 96	236	Lysine
	— 1	159	143	— 66	— 15	236	Methionine
	25	221	196	— 44	40	108	Histidine
	2	144	105	— 12	25	175	Threonine
	14	185	145	— 127	— 7	184	Valine
	4	111	151	— 60	45	178	Leucine
	0		229	— 110	11	175	Isoleucine
			143	— 49	35	137	Phenylalanine
			137		151 [1]		Arginine
Ave.	0	174	162	— 68	7	176	
S.E.	± 20	± 15	± 13	± 13	± 15	± 15	

[1] The value for the arginine-deficient animal was excluded from the average.

values for the animals fed the 4E diet for the first 10 days, column 2 the values for the animals fed the Cas diet for the same period. In column 3 are the gains of the animals fed the complete amino acid mixture (ration A) for the first 10 days following depletion. Column 4 contains the data for these same animals during the second 10-day period (10–20) during which they were fed a diet lacking in one of the amino acids. The missing amino acid is indicated in the extreme right-hand column. The fifth column contains data on the animals paired with those represented in the preceding 2 columns and fed the deficient amino acid ration in the first 10 days; column 6 contains the data for the same animals when fed ration A in the second 10-day period.

Rats on the 4E diet for the first experimental period (0 to 10 days) had a mean change of 0 ± 20 mg in their T.C.S.P. Over a similar 10-day period the 7 animals on the Cas diet gained 174 ± 15 mg of protein in their circulation, almost doubling the amount which they had initially. With these values we can compare the performance of rats fed the other experimental diets.

All animals fed ration A during the first 10-day period gained considerable quantities of total circulating serum protein. The mean gain was 162 mg with a standard error of ± 13 mg. In the second 10-day period when these same animals were fed diets lacking one of the essential amino acids they all lost serum protein. The smallest loss was 12 mg, the largest 127 mg, the average 68. It is evident that after an initial feeding period the lack of one essential amino acid induces a greater loss in serum protein than in the animal at the end of a long period of protein depletion. The reason for this is not clear. It may be due to an increased metabolic rate related to the repletion of body proteins and enzyme systems during the preceding 10-day period.

When the initial diets lacked one of the essential amino acids, excluding arginine, diet consumption was markedly reduced and there were minor losses or gains of total circulating serum proteins of the order of those observed in the animals

on the 4E diet. There was one exception, the animal fed ration A minus lysine apparently lost 96 mg of protein. The mean change for the group of 9 animals was + 7 ± 15 mg. These same animals in the second 10-day period eating ration A gained on the average 176 ± 15 mg of protein. The smallest gain recorded was 108 mg by the rat fed the histidine deficient diet during its initial period.

The data (tables 2 and 3) on the animals used to test the indispensability of arginine show a striking difference from the data on the preceding 9 amino acids. The rat on the diet completely lacking arginine gained 151 mg of serum protein

TABLE 3

Total circulating serum protein and total circulating erythrocyte volume values for animals on diets lacking arginine and their controls.

RAT NO.	DIET	TOTAL CIRCULATING SERUM PROTEIN				TOTAL CIRCULATING RED CELL VOLUME			
		Day 0	Day 10	Day 20	Day 30	Day 0	Day 10	Day 20	Day 30
		mg	*mg*	*mg*	*mg*	*ml*	*ml*	*ml*	*ml*
154–2	Ration A	256	393	479	536	3.6	6.1	6.9	6.4
255–1	Ration A	257	456	519		3.6	5.7	6.3	. .
153–4	Minus arginine	282	433	488	559	3.3	4.8	6.8	6.2
247–7	Ration C	246	424	440		3.3	5.3	5.6	
247–3	Ration C	259	429	449		3.4	5.3	5.3	

in the first 10-day period. Its control on ration A gained 137 mg. In this instance the diets were not interchanged. In the second 10-day period the animal lacking arginine gained an additional 55 mg. After 30 days the test animal and its control had almost identical T.C.S.P. and T.C.E.V. values.

Data on the behavior of the total erythrocyte mass are presented in table 4. With no protein in the diet there was an average loss in 10 days of 0.1 ± 0.2 ml in total circulating erythrocytes. With 9% casein there was an average gain of 2.0 ± 0.3 ml of red blood cells. On ration A there was a gain of 2.4 ± 0.1 ml in the first 10-day period. For the animals fed diets lacking one of the following: tryptophane, lysine, methio-

nine, histidine, threonine, valine, leucine, isoleucine, and phenylalanine, there were variable gains, or losses. With methionine and threonine absent the gains were 0.9 and 0.8 ml, respectively, not significantly greater than the greatest apparent gain (+ 0.7 ml) in the group of animals on the diet completely lacking protein. Also their paired controls fed the same diets lacking methionine and threonine in the second 10-day period had the largest losses in circulating erythrocyte

TABLE 4

Gains or losses in total circulating red cell volume.

RATION	4 E	CAS	RATION A	RATION A MINUS	RATION A MINUS	RATION A	
PERIOD (days)	0–10	0–10	0–10	10–20	0–10	10–20	
	ml	*ml*	*ml*	*ml*	*ml*	*ml*	*The missing amino acid*
	— 0.7	2.4	2.1	— 0.7	0.1	1.5	Tryptophane
	— 0.3	1.7			0.2	1.1	Lysine
	— 0.3	1.5	2.7	— 1.1	0.9	1.6	Methionine
	— 0.4	2.3	1.9	— 0.2	— 0.2	1.3	Histidine
	0.0	2.4	2.8	— 0.9	0.8	1.9	Threonine
	0.5	2.5	2.0	0.5	0.2	1.8	Valine
	0.7	1.5	1.9	— 0.1	— 0.4	1.3	Leucine
	— 0.2	.	3.0	— 0.6	0.4	1.9	Isoleucine
			2.8	— 0.6	0.3	1.4	Phenylalanine
			2.5		1.5 [1]		Arginine
Ave.	— 0.1	2.0	2.4	— 0.5	0.3	1.5	
S.E.	± 0.2	± 0.3	± 0.1	± 0.2	± 0.1	± 0.1	

[1] The value for arginine-deficient animal was excluded from the average.

volume of all the group. All animals with the exception of the one lacking valine which were fed ration A minus one amino acid in the second experimental period, lost circulating erythrocytes. With valine absent there was a slight apparent gain of + 0.5 ml. The animal lacking arginine again performed quite differently, gaining 1.5 ml of erythrocytes in the first 10-day period. This is somewhat less than the smallest gain of 1.9 ml or than the mean gain of 2.4 ml among the

animals on ration A in the first period. But at 20 and 30 days the gain of T.C.E.V. of the arginine deficient animal equaled that of its paired control (see table 3).

The evidence from the weight gains, food consumption, gains of serum protein and hemoglobin described above, demonstrated that arginine was dispensable for the recovery of the hypoproteinemic rat. Therefore ration C was prepared and fed to 2 animals as described (Frazier et al., '46). The total circulating serum protein and erythrocyte volume changes are tabulated in table 3. The rats fed 9 "essential" amino acids (arginine absent) fabricated serum proteins about as well as the animals fed 16 amino acids over a 10-day interval. Red blood cell volumes in these animals equaled those of the rats on the casein diet but were poorer than those of the animals on ration A. The difference is not statistically significant with so small a number of animals.

DISCUSSION

Several workers have demonstrated that protein hydrolysates can maintain nitrogen balance and induce increases in the concentration of serum proteins in adult humans, children and dogs. Only one group of workers (Madden et al., '43, '44, '45) has systematically attempted to determine the amino acids essential for the production of serum proteins. This group has shown that in the protein-depleted dog the 10 essential amino acids of Rose ('38) will induce and maintain the abundant formation of plasma proteins, and maintain positive nitrogen balance for long periods of time. The deletion of single essential amino acids from their mixtures led in some cases to negative nitrogen balances and weight loss, with continued plasma protein production, in others to sharp decreases in protein production with continued positive nitrogen balances. The omission of arginine (Madden et al., '43) from the diet caused the least disturbance of any of the "essential" amino acids they had tested to that time. There was a slow fall in serum protein production but nitrogen balance was maintained for the 2-week period. This is not dissimilar from the

data presented above except in the behavior of the serum proteins. It is apparent that the rat receiving no arginine in his diet over a period of 30 days gained weight, total circulating protein and erythrocytes at a rate comparable, in fact, almost identical with its paired control. It would appear, therefore, that arginine is not essential for the production of serum proteins or erythrocytes in the adult hypoproteinemic rat. This is borne out by the data on the animals fed 9 "essential" amino acids (arginine absent) as practically the sole amino nitrogen source.

Diet consumption with these amino acid mixtures has been described in paper I of this series. One must, of course, consider the total nitrogen intake in relation to the gains or losses of protein and erythrocytes as well as body weight. As noted in the preceding publication, with a deficiency of one of the essential amino acids diet consumption followed a particular pattern from day to day and was far below that of the complete mixture except in the absence of arginine. Because of the low diet consumptions it is difficult to say exactly what the losses in serum proteins mean. One can calculate the ratio of output of serum protein to intake of protein in those instances where there was a positive output. Thus the mean output of serum proteins in mg gained per gm of protein eaten was 13.2 with a range of 8.1 to 16.1 for the animals on the complete mixture for the first 10-day period. Compared with this the animal lacking tryptophane had a ratio of 4.2, lacking histidine 4.6, threonine 7.3, leucine 12.8, isoleucine 3.3, phenylalanine 12.1. The other animals had negative values for this ratio which, of course, have no real significance. In the case of the individual lack of tryptophane, histidine, threonine and isoleucine this ratio is well below the average for animals fed ration A and less than the performance of the poorest animal in that group. But in the case of the leucine and phenylalanine deficiencies the ratio of production to intake approximates that of the complete diets despite the extremely low intake (3.5 and 2.9 gm of protein ($N_2 \times 6.25$)). In view of the small number of animals on each

deficient diet and the curious behavior of the animals with respect to the diet consumption an evaluation of the biological effects of the deficiency of single essential amino acids must await further study.

SUMMARY AND CONCLUSIONS

Protein-depleted rats can synthesize new plasma protein and erythrocytes on a diet in which the sole source of amino nitrogen is a mixture of crystalline amino acids. The mixture includes tryptophane, lysine, methionine, histidine, threonine, valine, leucine, isoleucine, phenylalanine, and arginine, in relative proportions equivalent to those found by chemical analysis of casein. Small amounts of 6 non-essential amino acids were added to bring the nitrogen level to the equivalent of 9% protein (N $\times$ 6.25). Moreover, synthesis occurs to an extent comparable to that produced by a diet containing casein at an equivalent nitrogen level. With synthetic diets identical to the above, but lacking one of the above mentioned amino acids other than arginine there is poor diet consumption and no significant gain in serum protein, just as with the complete absence of protein from the diet. The same is true concerning the production of erythrocytes. On a synthetic amino acid diet entirely lacking arginine a rat can synthesize proteins and erythrocytes to an extent equalling that produced by casein or amino acid mixtures at equivalent nitrogen levels and containing all essential amino acids including arginine. Furthermore, the first mentioned 9 amino acids alone can act as a nitrogen source capable of inducing the formation of serum proteins to an extent comparable to that found with the mixture of 16 amino acids (ration A or "synthetic casein") or the natural protein. Therefore, it can be said that 9 amino acids only are indispensable in the hypoproteinemic rat for the construction of serum protein and erythrocytes.

LITERATURE CITED

BARBOUR, H. G., AND W. F. HAMILTON 1926 The falling drop method for determining specific gravity. J. Biol. Chem., *69;* 625.

BENDITT, E. P., R. L. STRAUBE AND E. M. HUMPHREYS 1946 The determination of total circulating serum proteins and erythrocyte volumes in normal and protein depleted rats. Proc. Soc. Exp. Biol. Med., *62;* 189.

FRAZIER, L. E., R. W. WISSLER, C. H. STEFFEE, R. L. WOOLRIDGE AND P. R. CANNON 1946 Studies in amino acid utilization. I. The dietary utilization of mixtures of purified amino acids in protein-depleted adult albino rats. J. Nutrition, *33;* 65.

MADDEN, S. C., F. W. ANDERSON, J. C. DONOVAN AND G. H. WHIPPLE 1945 Plasma protein production influenced by amino acid mixtures and lack of essential amino acids. J. Exp. Med., *82;* 77.

MADDEN, S. C., J. R. CARTER, A. A. KATTUS, L. L. MILLER AND G. H. WHIPPLE 1943 Ten amino acids essential for plasma protein production effective orally and parenterally. J. Exp. Med., *77;* 277.

MADDEN, S. C., R. R. WOODS, F. W. SHULL AND G. H. WHIPPLE 1944 Amino acid mixtures effective parenterally for long continued plasma protein production and casein digests compared. J. Exp. Med., *79;* 607.

METCOFF, J., AND C. B. FAVOUR 1944 Determination of blood and plasma volume partition in the growing rat. Am. J. Physiol., *141;* 695.

ROSE, W. C. 1938 The nutritive significance of the amino acids. Physiol. Rev., *18;* 109.

RELATIVE ACTIVITY OF THE TOCOPHEROLS IN CURING MUSCULAR DYSTROPHY IN RABBITS [1]

E. L. HOVE AND PHILIP L. HARRIS
Research Laboratories, Distillation Products, Inc., Rochester, New York

THREE FIGURES

(Received for publication September 12, 1946)

Nutritional muscular dystrophy in herbivora can be prevented or cured by α-tocopherol, but we can find no reports on the effectiveness of β- or γ-tocopherol relative to the alpha form. This information is essential for 2 reasons: First, the development of a bioassay using dystrophic rabbits or guinea pigs requires such knowledge, and second, the comparative physiology of vitamin E action may be better understood when the relative activity is known of the several tocopherols in curing such apparently unrelated syndromes as fetal resorption of rats and dystrophy of rabbits.

The high urinary excretion of creatine associated with muscular dystrophy rapidly falls to normal when α-tocopherol is given. This observation led Mackenzie and McCollum ('40) and Eppstein and Morgulis ('42) to suggest this as the criterion in a semi-quantitative bioassay procedure for vitamin E. A complicating factor demonstrated by Mackenzie et al. ('41) is the sensitivity of the α-tocopherol requirement of the rabbit to variations in intake of the cod-liver oil used to accelerate the onset of dystrophy. Is this effect due to the highly unsaturated fat acids in the oil? And, if so, would the highly unsaturated fat acids in vegetable oils have a similar action?

[1] Communication No. 101 from the Laboratories of Distillation Products, Inc., Rochester, N. Y.

EXPERIMENTAL

Young male New Zealand White rabbits weighing 600 to 900 gm were transferred gradually over the course of 10 days from a complete diet of ground rabbit chow to a vitamin E-low ration. The composition of the diets is given in table 1. Symptoms of muscular weakness began to appear in 7 to 18 days on either diet 38 or diet 381.

In the work to be reported creatinuria has been adopted as the criterion for the estimation of the severity of muscular dystrophy. Creatine and creatinine were determined daily

TABLE 1

Percentage composition of diets.

	DIET		
	381	38	38-A
Commercial casein	12	15	15
Dry skim milk	15	..	..
Dextrin	36	51	51
Sucrose	10		
Brewer's yeast	8	10	10
Salt mixtures	4	6	6
Cellulose	7	10	10
Lard	6	6	6
Cod-liver oil	2	2	..

by the method of Folin ('14), adapted to the Evelyn colorimeter. Creatinuria is expressed numerically as the ratio of the total creatinine (creatine plus creatinine) to preformed creatinine, rather than as the absolute values for 24-hour excretion. This is essentially the method used by Eppstein and Morgulis ('42) and has the advantage of not requiring a large number of metabolism cages. The procedure assumes that the creatinine excretion is constant and is unaffected by the dystrophic condition. This is not strictly true, since Mackenzie and McCollum ('41) have shown variations in the creatinine excretion curve ranging from a maximum of about 30 mg daily during dystrophy to about 22 mg daily in the normal rabbit. However, with creatine values of 70 to 90 mg

during dystrophy, falling to less than 10 mg daily in the normal rabbit, this slight creatinine variation does not seriously interfere with the ratio method of following the dystrophic condition.

A numerical creatinuria-ratio value of "1" indicates no creatine. A value of less than 1.3 is considered normal. When the ratio value has risen to about 4, a single dose of a curative test substance is given and the creatinuria ratios are determined daily until dystrophy has again set in. A single rabbit is often used for as many as 5 determinations, but deaths are common since it is difficult to determine when a rabbit has gone beyond the response stage. Occasionally it appears that supplement administration precipitates a complete, spastic "paralysis" in which the animal's body is entirely nonflexible and it is unable to raise its head to eat. This condition lasts for several days before death. α-Tocopherol given by mouth or subcutaneous injection of α-tocopheryl phosphate is of no benefit.

The response resulting from a test substance is taken as the number of days between dosage and the time when the creatinuria ratio has reached the same level as at the dosage point. This procedure eliminates some of the effect of creatinine variation, since the animals presumably have the same degree of dystrophy at both time points.

A typical example of the growth and creatinuria response to α-tocopherol is shown in figure 1. A certain degree of correlation can be noted between the growth effects and the creatinuria ratio responses. As pointed out by Mackenzie et al. ('40), creatinuria is the most dependable symptom of vitamin E deficiency in rabbits and precedes both the weight change and the physical symptoms. Although creatinuria may result from causes other than vitamin E deficiency, we feel that this possibility can be ignored here because all rabbits are subjected to at least 1 cure with α-tocopherol.

The tocopherol compounds to be tested were dissolved in U.S.P. olive oil, in such concentrations that between 4 and 8 drops (0.1 to 0.2 gm) delivered from a specially calibrated

dropper would furnish the desired quantity. Calibration was made by weight and by the Emmerie-Engel reaction for fat-soluble reducing substances. The materials tested were natural d,α-, β-, and γ-tocopherol prepared by Dr. Karl Meng in this laboratory, the synthetic dl,α- and γ-tocopherols,[2] and d,α-tocopheryl phosphate of 70% purity used in water solution.

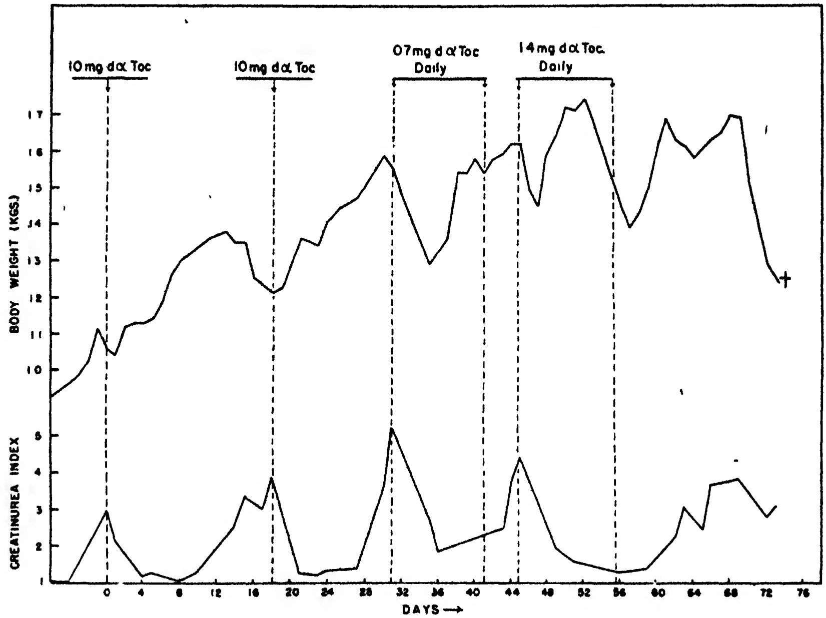


Fig. 1 Typical growth responses and changes in creatinurea index induced by oral administration of α-tocopherol. In some instances vitamin E was fed in a single dose and in others it was fed daily for 2 weeks.

Response to d,α-tocopherol

Several levels of d,α-tocopherol varying from about 2 to 24 mg were fed in single doses to dystrophic rabbits. The creatinuria ratio curves were determined as outlined in the previous section. Dosages are expressed in terms of milligrams per kilogram of body weight at the time of dosage. The responses (days cured) were obtained from the ratio curves.

[2] Merck.

From the results tabulated in table 2 it appears that the responses are a linear function of the logarithm of the dose (mg/kg body weight); on diet 38 the average constant, K, (response/log dose) is 12.5 ± 3.1 (S.D.) while on diet 381 the value is 15.7 ± 1.8 (S.D.). These values correspond with

TABLE 2

Dose-response relations on feeding single doses of d,a-tocopherol to dystrophic rabbits.

BODY WEIGHT	DOSE	LOG DOSE	RESPONSE	K
kg	*mg/kg*		*days*	$\frac{\text{days cured}}{\text{log dose}}$
		A. Diet 38		
0.60	3.54	0.549	5.8	10.6
0.65	3.28	0.516	6.7	13.0
0.64	6.65	0.823	9.0	10.9
0.80	5.30	0.724	13.0	17.9
0.71	6.00	0.778	8.0	10.3
0.55	15.40	1.188	10.5	8.9
0.60	14.20	1.152	13.3	11.5
0.70	12.1	1.083	18.7	17.3
0.95	21.2	1.326	16.6	12.5
0.75	22.6	1.354	21.4	15.8
0.80	21.2	1.326	11.5	8.7
				12.5 ± 3.1 (S.D.)
		B. Diet 381		
1.08	22.20	1.346	24.0	17.8
1.06	9.42	0.974	15.0	15.4
1.21	8.25	0.917	12.2	13.3
1.37	14.60	1.164	17.5	15.0
1.41	4.26	0.629	9.5	15.1
1.07	18.70	1.272	22 8	17.9
				15.8 ± 1.8 (S.D.)

an average daily curative dose of 1.2 and 1.1 mg α-tocopherol, respectively. Diet 38 has been used for the remainder of the work to be reported, but it appears that diet 381 has many points of superiority, chief of which is the smaller coefficient of variation in response and the better growth and general health obtained.

Since the apparent curative dose of α-tocopherol is about 1.1 mg/kg daily, a dystrophic rabbit was given a suboptimal dosage of 0.7 mg of d,α-tocopherol daily for 11 days. As shown in figure 1 the creatinuria declined but was not completely cured. After cessation of dosage the creatinuria rose almost immediately. A larger daily dose (1.4 mg) of α-tocopherol brought about a more effective cure.

TABLE 3

Activity of various tocopherols in terms of d,α-tocopherol equivalence.

BODY WT. OF RABBIT	DOSE	LOG DOSE	DAYS CURED	d,α-TOCOPHEROL EQUIVALENCE	
kg	*mg/kg*			*mg/kg*	%
			dl,α-Tocopherol		
1.05	16.0	1.204	13.0	11.0	68.8
0.95	11.8	1.072	8.6	4.9	41.5
0.80	17.5	1.243	15.4	17.0	97.7
1.20	9.4	0.973	8.3	4.7	48.9
0.92	12.2	1.086	12.2	9.5	77.9
1.30	10.8	1.033	13.0	11.0	101.9
1.04	10.8	1.033	14.6	14.8	136.1
				Average	81.8 ± 12.4 (S.E.)
			d,β-Tocopherol		
0.75	14.7	1.167	7.7	4.2	28.5
0.80	14.0	1.146	7.9	4.3	30.7
				Average	29.6
			d,γ-Tocopherol		
0.60	33.4	1.524	7.7	4.2	12.6
0.65	30.8	1.489	10.5	7.1	23.1
0.60	16.7	1.223	7.2	3.8	22.8
				Average	19.5
			dl,γ-Tocopherol		
0.75	40.0	1.602	3.0	1.7	4.3
0.60	83.2	1.920	8.9	5.2	6.2
1.25	40.0	1.602	7.6	4.1	10.2
				Average	6.9

Response to other tocopherols

Harris, Jensen, Joffe and Mason ('44) noted that dl,α-tocopherol was only 66% as active as d,α-tocopherol in preventing resorptive sterility in rats. A similar difference in the anti-dystrophy potency of these 2 substances has been observed, as shown in table 3; the racemic mixture has, on the average, 82% of the activity of the natural enantiomorph. However, the variations are large. By the statistical "t" test of Fisher the difference is only significant at a level of $P = 0.2$ (80 chances in 100 that the difference is significant). The average K value of 12.5 as obtained is table 1 was used in calculating the results since these rabbits were on diet 38.

The natural d,γ-tocopherol is almost 3 times as active as the synthetic dl,γ-tocopherol in curing dystrophy (table 3). The synthetic γ- has about 8.5% of the activity of synthetic α-, and the natural γ- has about 20% of the activity of the natural α-. A small part of the difference between natural and synthetic γ-tocopherols can be attributed to α-tocopherol remaining in the former.

The natural d,β-tocopherol shows about 30% of the activity of the d,α-tocopherol.

In general, the activity of the various forms of tocopherol shows much the same relationship in the cure of dystrophy in rabbits as in the prevention of resorption in pregnant rats (Harris et al., '44).

Response to d,α-tocopheryl phosphate administered orally and parenterally

Injections of α-tocopherol or α-tocopheryl acetate are of little or no benefit in curing muscular dystrophy in the rabbit (Mackenzie and McCollum, '41; Eppstein and Morgulis, '41). Injections of d,α-tocopheryl phosphate, on the other hand, are somewhat more active than the oral administration of dl,α-tocopheryl acetate (Eppstein and Morgulis, '42).

We have fed and injected d,α-tocopheryl phosphate into dystrophic rabbits with the following results: Orally the

tocopheryl phosphate was 35% more potent than equivalent doses of d,α-tocopherol. When the phosphate was injected intramuscularly cures were obtained, but the creatinuria ratio curves were of such a character as to make quantitative interpretation difficult. Three of these curves are shown in figure 2.

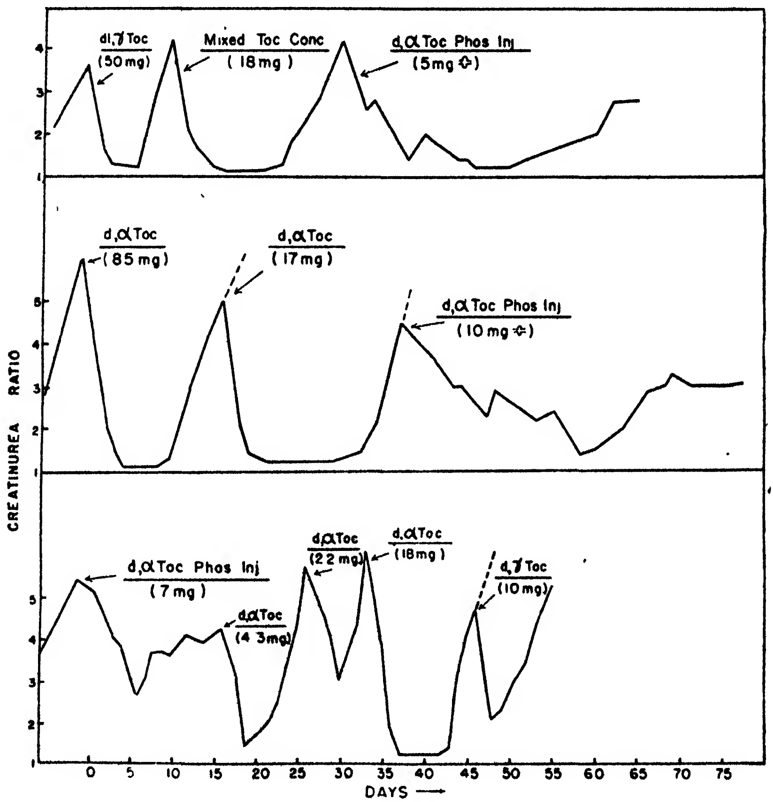


Fig. 2 Typical response, creatinurea ratios, obtained in rabbits injected with d,α-tocopheryl phosphate.

It will be noted that the urinary creatine falls to a normal level much more slowly than after oral tocopherol administration. Similarly the recurrence of dystrophy is sluggish. In the case of the first 2 animals in figure 2, the gain in body weight during the 30 and 34 days following tocopheryl phos-

phate injection was 300 and 360 gm, respectively. The rabbits then began to lose weight with little or no change in their level of creatine excretion. After becoming extremely emaciated they died by the end of the second month without again developing more than a trace of muscular weakness. There is no way in which their creatinuria ratio curves can be quantitatively interpreted according to the formula used in the previous section. If the recurrence of dystrophy is taken as the point where weight loss sets in, the activity of the injected tocopheryl phosphate may be calculated at 50 and 53 times more active than d,α-tocopherol given orally, and about 6 times more active in the case of the third rabbit of figure 2.

Effect of unsaturated fats on production of dystrophy

The vitamin E requirement of several species is known to be increased by the inclusion of unsaturated fats in the diet. Gottlieb et al. ('43) showed this for rats using tocopherol-free cottonseed oil. Dam ('44) studied the effect of highly unsaturated fractions of hog-liver fat on the vitamin E needs of chickens. Mackenzie and McCollum ('41) have shown that cod-liver oil increases the vitamin E requirement of rabbits and aggravates the dystrophic condition.

Figure 3 illustrates the rate of onset of dystrophy in rabbits as influenced by unsaturated fats, using the creatinuria ratio as the criterion. For this series 38-A was used (diet 38 with the cod-liver oil omitted). Young rabbits were transferred to this diet in the usual way. The various oils tested were fed daily from a hypodermic syringe in quantities of 1.2 ml. All oils were fortified with a concentrate of vitamins A and D to the level of cod-liver oil. The control receiving no oil was fed an equivalent amount of the A and D concentrate by dropper weekly. The oils used were freed of their tocopherols by molecular distillation.[3]

From the results shown in figure 3 it is apparent that the vitamin-free soybean and corn oils and corn oil plus 0.1%

[3] This was kindly done for us by Mr. E. Barnitz of this laboratory.

hydroquinone were as effective as cod-liver oil in inducing dystrophy. The feeding of the oils was stopped on the sixteenth day. It is interesting to note that the creatine excretion dropped toward normal when the oil was stopped, showing more clearly a direct relation of the oils to the condition of dystrophy. After a temporary recession the creatinuria set in again without restitution of the oils.

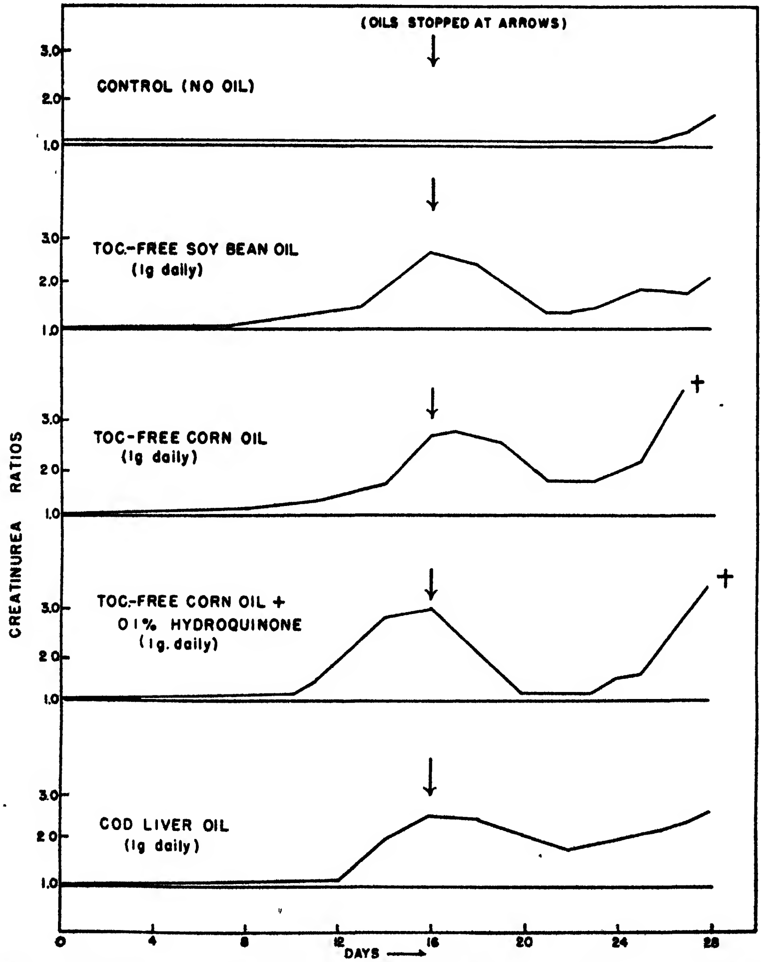


Fig. 3 Changes in creatinurea ratios of rabbits fed 1.2 ml daily of various oils. Unsaturated vegetable fats are as effective as cod liver oil in inducing creatinurea (muscle dystrophy).

DISCUSSION

The natural d,α-, β-, and γ-tocopherols show anti-creatinuria activity in the ratio of 1.0, 0.3, and 0.2, respectively. The synthetic dl,α- and γ-tocopherols are less active than the natural forms. These relative activities are of approximately the same order and degree as has previously been found for the prevention of fetal resorption in rats (Harris et al., '44). The relative activities are also in the same order as their antioxidant potencies in vitro, as determined by Hove and Hove ('44).

The cure of the creatinuria of dystrophic rabbits following injection of d,α-tocopheryl phosphate shows an interesting situation. It appears that the injected tocopheryl phosphate is less readily available to the animal than tocopherol given orally. This is shown by the slow fall of creatine excretion. However, the activity is maintained over a much longer period than would be expected, and recurrence of dystrophy occurs very slowly. The animals may be said to be in a chronic state of dystrophy. Possibly these results indicate that the phosphate is not the physiologically necessary form of the vitamin. An alternate explanation is that the phosphate becomes bound to protein near the site of injection and is liberated slowly over a long period of time. A search for tocopheryl phosphate esterases in animal tissues might throw some light on this situation.

The interrelation of unsaturated fats and tocopherol has been emphasized by showing that the dystrophy-inducing properties of cod-liver oil are shared by tocopherol-free soybean oil and corn oil. The reverse of this interrelation, wherein tocopherols protect or spare essential unsaturated fat acids has recently been shown by Hove and Harris ('46).

In its present form the cure of dystrophy in rabbits does not offer special advantage as a bioassay procedure for vitamin E. Although it is more rapid, it lacks precision and is somewhat more laborious than the fetal resorption method using rats.

SUMMARY

1. The relative activity of natural α-, β-, and γ-tocopherols in the cure of creatinuria associated with muscular dystrophy in rabbits has been found to be 100, 30, and 20, respectively.

2. The minimum curative dose of natural (d) α-tocopherol was 1.1 mg per kilo of body weight per day. For synthetic (dl) α-tocopherol the minimum curative dose was 1.4 mg per kilo per day.

3. The anti-dystrophy potency of synthetic dl,γ-tocopherol was about 30% that of natural d,γ-tocopherol.

4. d,α-Tocopheryl phosphate injected intramuscularly into dystrophic rabbits brings about a slower but longer-lasting cure. The possible significance of this is discussed.

5. Tocopherol-free corn and soybean oils are as effective as cod-liver oil in accelerating the onset of nutritional muscular dystrophy.

LITERATURE CITED

DAM, H. 1944 Toxicity of hog liver fatty acids to chicks fed a vitamin E deficient diet. J. Nutrition, *28:* 297.

EPPSTEIN, S. H., AND S. MORGULIS 1941 The minimum requirement of rabbits for dl, alpha tocopherol. J. Nutrition, *22:* 415.

——— 1942 Factors influencing the onset and cure of nutritional muscular dystrophy. J. Nutrition, *23:* 473.

FOLIN, O. 1914 On the determination of creatinine and creatine in urine. J. Biol. Chem., *17:* 469.

GOTTLIEB, H., F. W. QUACKENBUSH AND H. STEENBOCK 1943 The biological determination of vitamin E. J. Nutrition, *25:* 433.

HARRIS, P. L., J. L. JENSEN, M. JOFFE AND K. E. MASON 1944 Biological activity of natural and synthetic tocopherols. J. Biol. Chem., *156:* 491.

HOVE, E. L., AND P. L. HARRIS 1946 Covitamin studies. V. The interrelation of alpha-tocopherol and essential unsaturated fat acids. J. Nutrition, *31:* 699.

HOVE, E. L., AND Z. HOVE 1944 Effect of temperature on the relative antioxidant activity of α-, β-, and γ-tocopherols. J. Biol. Chem., *156:* 623.

MACKENZIE, C. G., AND E. V. MCCOLLUM 1940 The cure of nutritional muscular dystrophy in the rabbit by alpha-tocopherol and its effect on creatine metabolism. J. Nutrition, *19:* 345.

——— 1941 Effect of oral and parenteral administration of vitamin E on creatinuria and symptoms of dystrophic rabbits. Proc. Soc. Exp. Biol. Med., *48:* 642.

MACKENZIE, C. G., J. B. MACKENZIE AND E. V. MCCOLLUM 1941 Uncomplicated vitamin E deficiency in the rabbit and its relation to the toxicity of cod liver oil. J. Nutrition, *21:* 225.

THE ABSORPTION OF RADIOACTIVE IRON BY CHILDREN 7–10 YEARS OF AGE [1]

WILLIAM J. DARBY, PAUL F. HAHN, MARGARET M. KASER, RUTH C. STEINKAMP, PAUL M. DENSEN AND MARY B. COOK

Departments of Medicine, Biochemistry, and Preventive Medicine and Public Health, Vanderbilt University School of Medicine; the Tennessee Department of Public Health; and the Nashville School Health Service, Nashville

TWO FIGURES

(Received for publication September 16, 1946)

Efforts to assess the nutriture of children with respect to iron have usually been limited to studies of hemoglobin concentration and of dietary intakes. Both of these methods have recognized limitations and the interpretation of the data obtained is often controversial. Thus, hemoglobin concentrations below arbitrary limits varying from 10 to 14 gm per 100 ml have been interpreted as signifying anemia (Committee on Diagnosis and Pathology of Nutritional Deficiencies, '43) and dietary intakes less than the Recommended Dietary Allowances have been cited as evidence of iron deficiency states, although these Recommended Allowances were purposely set at a high level and the estimates of allowances for children were based on rather meager data (Food and Nutrition Board, National Research Council, '45).

These considerations have prompted us to organize a pilot study of the absorption of radioactive iron by a group of school children (Darby, Hahn, Steinkamp and Kaser, '46).

[1] This study was supported by grants from the Nutrition Foundation, Inc., the International Health Division of The Rockefeller Foundation, and the Tennessee Department of Public Health.

The data accumulated in this study have not revealed the presence of any considerable degree of iron deficiency among the children studied, but the study has given a new approach to the problem of iron requirements. This study has demonstrated for the first time the feasibility of employing isotopes in large-scale surveys of the nutriture of populations and has led to the institution of a more extensive similar investigation of the iron requirements during pregnancy (Vanderbilt Cooperative Study of Maternal and Infant Nutrition, to be published)

EXPERIMENTAL

Two grade schools for white children in Nashville, Tennessee, were selected to obtain the extremes of economic groupings. School C served a low economic area, School R a high economic district. Some characteristics of the two school districts are compared in the data of table 1 derived from the sixteenth census of the U.S. ('42). The total number of subjects examined were 259 and comprised 88% of the second and third grade pupils in School R and 92% of the pupils in the same grades in School C. All studies were made between January 22 and March 31, 1945.

The following observations were made on each child: a physical examination, laboratory determinations on venous blood of total hemoglobin (Evelyn and Malloy, '38; Rubicon Company, no date), packed cell volume (Wintrobe, '33), serum albumin (micro-Kjeldahl procedure), serum vitamin A (Kaser and Stekol, '43) and ascorbic acid (Bessey, '38), and the calculation of a 7-day food intake record (Steinkamp, Robinson and Kaser, '45). Measurement of the radioactive iron absorption was completed as follows on 188 of the subjects,[2] 176 of whom were aged 7–10 years. A known aliquot of 2–3 mg of ferrous chloride containing Fe^{59}, 47-day half life, reduced

[2] The number of subjects upon whom the iron studies were completed is smaller than the total number of children examined because of unavoidable losses of samples, of inability to complete the studies on some children due to absenteeism, moving from the area, etc. There was no purposeful selection of the subjects for iron absorption studies and no evidence of the occurrence of unconscious selection.

with a slight excess of ascorbic acid, was given orally. This was dispensed in a cup of lemonade and the cup was then rinsed by a second serving of the beverage without added iron. The test dose was administered at least an hour before or after lunch. Two to 4 weeks later another 10-ml sample of venous blood was taken for estimations of the radioactivity

TABLE 1

Population and housing characteristics of school districts R and C, Nashville, Tennessee, according to the U. S. census of 1940.

CHARACTERISTIC	NUMBER OF INDIVIDUALS		PER CENT OF TOTAL	
	District R	District C	District R	District C
I Major occupation group				
Total in occupation group	2,162	4,271	100.0	100.0
Professional workers	311	57	14.4	1.3
Semi-professional workers	41	26	1.9	0.6
Proprietors, managers, officials	534	196	24.7	4.6
Clerical, sales, and kindred workers	884	742	40.9	17.4
Craftsmen, foremen, and kindred workers	101	728	4.7	17.0
Operators and kindred workers	51	1,360	2.4	31.8
Domestic service workers	152	349	7.0	8.2
Service workers, except domestic	66	355	3.0	8.3
Laborers	7	432	0.3	10.1
Occupation not reported	15	26	0.7	0.6
II Population by race				
Total	5,161	13,239	100.0	100.0
White	4,977	11,456	96.4	86.5
Colored	184	1,783	3.6	13.5
III Per cent of dwelling units owner-occupied			52.7	8.6
IV Median contract or estimated monthly rent of all dwelling units (dollars)	49.79	13.96		
V Per cent of dwelling units needing major repairs			2.5	18.6

of hemoglobin iron and, simultaneously, the packed cell volume and the hemoglobin concentrations were again determined. The estimations of radioactive iron were made as described elsewhere (Hahn, '45; Hahn, Jones, Lowe, Meneely and Peacock, '45).

From the packed cell volume and the body weight data the total red cell mass was estimated by the formula of Balfour, Hahn, Bale, Pommerenke and Whipple ('42):

$$\text{Body weight in kg} \times 80 \times \text{venous hematocrit reading} \times 0.75 = \text{Estimated cell mass}$$

The total radioactivity of the circulating hemoglobin iron was then calculated and the percentage absorption of radioactive iron estimated. In this estimation the two assumptions were made that most of the absorbed iron was converted to the iron of the hemoglobin of the circulating red cells and that the iron was not excreted. These assumptions have been demonstrated to be valid for adult iron deficient animals (Hahn and Whipple, '36; McCance and Widdowson, '37; Miller and Hahn, '40; Hahn, Bale, Ross, Hettig and Whipple, '40; Hahn, Bale, Hettig, Kamen and Whipple, '39; Cruz, Hahn and Bale, '42; Moore, Dubach, Minnich and Roberts, '44), although the validity has not been tested for the growing organism.

RESULTS AND DISCUSSION

That there existed a pronounced difference in the economic levels of these two groups of children is apparent from the data of table 1. The children in School R, the economically higher district, had more abundant intakes of calories, protein, vitamins, and minerals than did those of School C. Likewise they showed greater body weights for their ages and higher levels of serum ascorbic acid and carotene (table 2). By all measures the children of School R were abundantly nourished. Despite these differences, the differences in mean hemoglobin concentrations by sex for the ages 7 to 10 are but on the borderline of statistical significance (table 3). The differences by sex at the individual ages are significant only for 10-year old boys and 7-year old girls, although the numbers are small

TABLE 2

Comparison of mean nutritional assessments of children in schools B and C, Nashville, Tennessee, 1945.

	SCHOOL B			SCHOOL C		
ASSESSMENT	No. of children [1]	Sex	Mean ± S.E.	No. of children [1]	Sex	Mean ± S.E.
Per cent of standard weight	60	MF	105.0 ± 1.6	199	MF	97.2 ± 0.7
Calculated daily intake						
Calories	26	M	1931 ± 73	82	M	1842 ± 43
Calories	30	F	1850 ± 58	102	F	1745 ± 38
Protein (gm)	56	MF	68.2 ± 1.7	184	MF	59.5 ± 1.1
Calcium (mg)	56	MF	1193 ± 34	184	MF	889 ± 23
Iron (mg)	56	MF	11.7 ± 0.36	184	MF	10.8 ± 0.19
Total vitamin A (I.U.)	56	MF	6429 ± 436	184	MF	3793 ± 169
Ascorbic acid (mg)	56	MF	67.2 ± 3.7	184	MF	36.6 ± 1.6
Laboratory findings						
Serum ascorbic acid (mg/100 ml)	47	MF	0.814 ± 0.07	187	MF	0.493 ± 0.03
Serum carotene (μg/100 ml)	52	MF	152 ± 7.3	181	MF	96 ± 3.0
Serum vitamin A (I.U./100 ml)	50	MF	93 ± 3.6	172	MF	95.7 ± 2.3
Serum albumin (gm/100 ml)	46	MF	4.96 ± 0.037	190	MF	4.97 ± 0.022

[1] Some of the data on certain children were incomplete; hence, the variations in the numbers.

and there is a general trend toward slightly lower values in School C. We do not feel that the magnitude of these differences is great enough to indicate a physiologically important distinction between the 2 schools. It is apparent from figure 2 that there existed no consistent differences in the radioiron absorption of the children in the 2 schools. Hence, for purposes of further analyses we have combined the data on children from the two schools.

The mean hemoglobin values (table 3) are essentially identical with the means for children in elementary and secondary schools, preparatory boarding schools, and public schools in Great Britain in 1943 (Committee on Haemoglobin Surveys,

TABLE 3

Mean hemoglobin concentrations of children,[1] age 7–10 years, schools C and R, Nashville, Tennessee, 1945.

	SCHOOL R				SCHOOL C			
SEX	Boys		Girls		Boys		Girls	
AGE YEARS	Number	Mean ± S E.	Number	Mean ± S E	Number	Mean ± S E.	Number	Mean ± S.E.
		gm/100 ml		gm/100 ml		gm/100 ml		gm/100 ml
7	7	13.12 ± 0.156	12	13.33 ± 0.155	14	12.77 ± 0.221	20	12.70 ± 0.150
8	12	13.20 ± 0.223	12	13.18 ± 0.177	26	12.85 ± 0.186	40	12.98 ± 0.120
9	7	13.04 ± 0.163	2	12.95 ± 0.409	28	13.02 ± 0.115	28	12.80 ± 0.145
10	4	13.51 ± 0.402	0	.	19	12.85 ± 0.135	17	13.16 ± 0.119
7–10	30	13.19 ± 0.093	26	13.23 ± 0.119	87	12.89 ± 0.084	105	12.91 ± 0.066

[1] These data include all of the children studied in this age group. The data of the group upon which iron absorption studies were carried out did not differ significantly from these.

TABLE 4

Absorption of radioactive iron by children, schools C and R, Nashville, Tennessee, 1945.

AGE	7 YEARS		8 YEARS		9 YEARS		10 YEARS	
SEX	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls
Number	17	24	26	34	26	23	17	9
Mean absorption (per cent)	9.26 ± 1.41	7.75 ± 0.78	10.38 ± 1.22	15.84 ± 2.15	16.14 ± 1.01	16.89 ± 2.03	16.68 ± 1.92	14.5 ± 2.4
Median absorption (per cent)	7.75	7.00	8.25	12.00	15.00	13.75	17.75	12.75
Estimated total yearly iron requirement[1] (in mg)	70	67	72	108	152	120	130	163

[1] Data of Heath and Patek ('37).

'45), with the values recorded by Macy ('46) for healthy children, and with the mean levels of similarly aged children in British Columbia (Pett and Hanley, '46), and with those compiled by Wintrobe ('42). It seems unlikely that they reflect any appreciable degree of iron deficiency.

The adult with anemia due to iron deficiency exhibits a greater than usual absorption of iron by the method employed in this investigation (Balfour, Hahn, Bale, Pommerenke and Whipple, '42; Hahn, Jones, Lowe, Meneely and

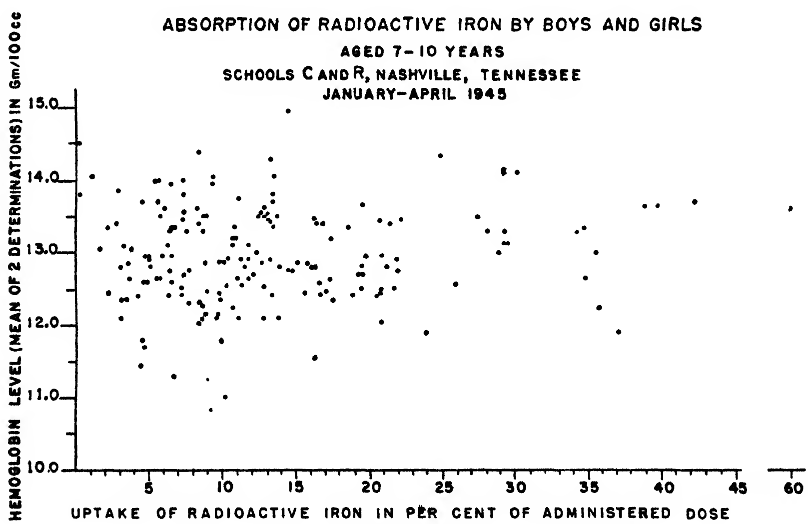


Figure 1

Peacock, '45; Moore, Dubach, Minnich and Roberts, '44; Darby, '46). Thus, hemoglobin concentration and iron absorption may both reflect a body deficit of this element. The lack of correlation between these 2 variables in this study (fig. 1) is interpreted by us as further evidence that the hemoglobin levels as a group were not limited by iron deficiency, and that the means and distributions of hemoglobin concentration found are probably satisfactory for this age group. This conclusion is also supported by the finding that the mean corpuscular hemoglobin concentration for the series was 31.9%

and 32.4% with standard deviations of 1.18 and 0.9, respectively, for Schools C and R. None of these children presented the physical signs of iron deficiency sometimes observed in adults (Darby, '46).

The mean calculated daily iron intake of these children was 11.7 ± 0.36 mg and 10.8 ± 0.19 mg, respectively, for Schools R and C. This level is to be compared with a mean intake of 9.0 mg and 10.7 mg for 7- to 8-year-olds and 10- to 12-year olds, respectively, concluded by Oldham, Roberts and Young ('45) to be adequate. Furthermore, the mean intakes of iron of these children approximated the Recommended Dietary Allowances (Food and Nutrition Board, '45) and the intakes considered by Johnston and Roberts ('42) to be satisfactory for a similar age group. The dietary studies, therefore, also indicated a generally satisfactory nutritional status of these children with respect to iron.

Analysis of the absorption data revealed a remarkable increase in the mean uptake of iron from the 7- to the 9-year groups (fig. 2). Although some of the distributions of absorption are somewhat skewed, the increase is apparent in both the means and medians of each age group and it occurs in the children of both schools. In the 8-year-old group there appeared a significant sex difference in the average per cent of iron absorbed (table 4). The girls absorbed a significantly higher percentage of the administered iron. Upon comparison of these mean absorption values with the estimates of Heath and Patek ('37) for the yearly iron requirements, it is seen that these differences in iron absorption parallel, in general, the estimated yearly increments of body iron. This parallelism is in agreement with the modern theory of iron metabolism (McCance and Widdowson, '37; Hahn, Bale, Lawrence and Whipple, '39). Thus, if the body absorbs iron only as it is needed, the increased requirement during periods of more rapid growth should be reflected in an increased absorption of iron from the gastrointestinal tract at this time. This same proportionality of absorption and need for iron is further illustrated by data on iron absorption during pregnancy (Bal-

four, Hahn, Bale, Pommerenke and Whipple, '42; Vanderbilt Cooperative Study of Maternal and Infant Nutrition, to be published), which indicate that there occurs an increased uptake of iron during the latter part of pregnancy.

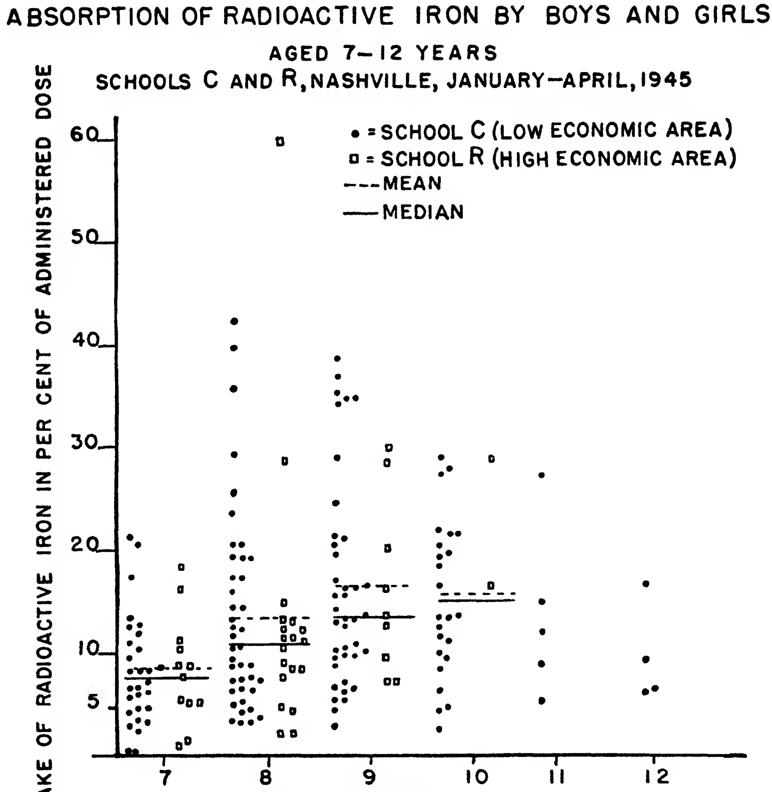


Figure 2

Since the quantity of iron given to these children is approximately that which one might obtain from an ordinary meal, it is possible to estimate from these mean absorption figures the actual mean intake of absorbable iron which is necessary to supply the yearly increment of body iron. This intake divided by 365 gives an estimate of the daily requirement

of absorbable iron. This calculation is illustrated by the formula:

$$\frac{\text{Total yearly gain in circulatory and parenchymal iron}}{365} \times \frac{100}{80} \times \frac{100}{\text{Per cent uptake}} = \text{Mean daily requirement of absorbable iron}$$

The factor $\frac{100}{80}$ is introduced as an allowance for the storage iron, of which the data of Heath and Patek do not take account, and is an approximation of the storage iron arrived at by Hahn ('37), from analyses of perfused dog tissues. Employing the estimates of Heath and Patek ('37) for the total yearly gain in circulatory and parenchymal iron and applying this calculation, one finds the following estimated mean daily requirements of absorbable iron: for boys 7, 8, 9 and 10 years old 2.6, 2.4, 3.2 and 2.7 mg, respectively; and for girls 7, 8, 9 and 10 years old 3.0, 2.3, 2.4 and 3.8 mg, respectively.

If one assumes that 50% of the dietary iron is absorbable it is obvious that this method of calculation results in a much lower estimate of requirements than the presently accepted allowances. Despite this, it is apparent that these calculations are maximal, for in them it has been assumed that all of the absorbed iron appears in the blood. A correction for iron deposited elsewhere would increase the estimate of absorption and, thereby, reduce the magnitude of the dietary requirement. It is emphasized that these estimates are for the mean dietary requirement of absorbable iron and at present that there exists no reliable means of estimating the quantity of absorbable iron in a dietary; hence, the requirement of total dietary iron cannot now be ascertained by this method. A similar estimate of yearly requirements of body iron based on body weight and the iron content of the tissues of lower animals (Hahn and Whipple, '36) indicates yearly increments approximately twice the magnitude obtained by Heath and Patek. More data on the iron content of human tissues are required before completely reliable estimations of requirements can be made by this method.

It is apparent that iron absorption data such as these may offer a valid means of determining requirements, especially

when quantitative estimates of absorbable iron in foodstuffs are available. The present pilot study demonstrates the possibilities inherent in this approach. The data lead us to suggest that the Recommended Dietary Allowances for iron for children of this age are liberal. It is probable that intakes lower than the present allowances would be adequate if one may assume that at least 50% of the dietary iron is absorbable.

SUMMARY

Determinations of iron absorption on 176 school children, aged 7–10 years, have indicated a mean uptake of 7.75 to 17.75% of a test dose of 2–3 mg of ferrous iron. The median intakes were slightly lower.

The uptake was found to be correlated with the estimated yearly increases of body iron during growth; it was not correlated in these children with other factors tested for, such as economic status of the group, dietary intake as estimated, the hemoglobin level, or general nutritive state.

A new method for the calculation of iron requirements is outlined.

It is estimated from these data that the mean daily requirement of absorbable iron is 2.3–3.8 mg for children of this age group.

It appears that the mean level of approximately 13 gm of hemoglobin in these children was not limited by iron deficiency.

ACKNOWLEDGMENTS

We wish to express our appreciation to Mr. W. A. Bass, Superintendent of Nashville City Schools and to the Nashville City School Board for granting us permission to carry out this study; to Miss Elizabeth Cawthorn, School Nurse; to the principals and teachers in the two schools who cooperated so wholeheartedly; to Miss Caroline Ashley and Mrs. Naomi Dziewiatkowski for technical assistance; to Mrs. Jo Haile Mayberry for clerical help; and, lastly, to the children and their parents for their splendid cooperation.

LITERATURE CITED

BALFOUR, W. M., P. F. HAHN, W. F. BALE, W. T. POMMERENKE AND G. H. WHIPPLE 1942 Radioactive iron absorption in clinical conditions; normal, pregnancy, anemia, and hemochromatosis. J. Exp. Med., *76;* 15.

BESSEY, O. A. 1938 A method for the determination of small quantities of ascorbic acid and dehydroascorbic acid in turbid and colored solutions in the presence of other reducing substances. J. Biol. Chem., *126;* 771.

Committee on Diagnosis and Pathology of Nutritional Deficiencies, Food and Nutrition Board 1943 Inadequate diets and nutritional deficiencies in the United States. Bull. Nat. Res. Council, no. 109, p. 26.

Committee on Haemoglobin Surveys, Medical Research Council 1945 Haemoglobin levels in Great Britain in 1943 (with observations upon serum protein levels). Special Report Series no. 252, Medical Research Council, London.

CRUZ, W. O., P. F. HAHN AND W. F. BALE 1942 Hemoglobin radioactive iron liberated by erythrocyte destruction (acetylphenylhydrazine) promptly reutilized to form new hemoglobin. Am. J. Physiol., *135;* 595.

DARBY, W. J. 1946 The oral manifestations of iron deficiency. J. Am. Med. Assoc., *130;* 830.

DARBY, W. J., P. F. HAHN, R. C. STEINKAMP AND M. M. KASER 1946 Absorption of radioactive iron by school children. Fed. Proc., *5;* 231.

EVELYN, K. A., AND H. T. MALLOY 1938 Microdetermination of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. J. Biol. Chem., *126;* 655.

Food and Nutrition Board 1945 Recommended dietary allowances, revised 1945. Reprint and Circular Series, National Research Council, no. 122, Washington.

HAHN, P. F. 1937 The metabolism of iron. Medicine, *16;* 249.

——— 1945 Radioactive iron procedures. Purification, electroplating, and analysis. Ind. Eng. Chem., Anal. Ed., *17;* 45.

HAHN, P. F., W. F. BALE, R. A. HETTIG, M. D. KAMEN AND G. H. WHIPPLE 1939 Radioactive iron and its excretion in urine, bile, and feces. J. Exp. Med., *70;* 443.

HAHN, P. F., W. F. BALE, E. O. LAWRENCE AND G. H. WHIPPLE 1939 Radioactive iron and its metabolism in anemia. J. Exp. Med., *69;* 739.

HAHN, P. F., W. F. BALE, J. F. ROSS, R. A. HETTIG AND G. H. WHIPPLE 1940 Radio-iron in plasma does not exchange with hemoglobin iron in red cells. Science, *92;* 131.

HAHN, P. F., E. JONES, R. C. LOWE, G. R. MENEELY AND W. PEACOCK 1945 The relative absorption and utilization of ferrous and ferric iron in anemia as determined with the radioactive isotope. Am. J. Physiol., *143;* 191.

HAHN, P. F., AND G. H. WHIPPLE 1936 II. Iron metabolism. Its absorption, storage and utilization in experimental anemia. Am. J. Med. Sci., *191;* 24.

HEATH, C. W., AND A. J. PATEK, JR. 1937 The anemia of iron deficiency. Medicine, *16;* 267.

JOHNSTON, F. A., AND L. J. ROBERTS 1942 The iron requirement of children of the early school age. J. Nutrition, *23*; 181.

KASER. M., AND J. A. STEKOL 1943 A critical study of the Carr-Price reaction for the determination of β-carotene and vitamin A in biological materials. J. Lab. Clin. Med., *28*; 904.

McCANCE, R. A., AND E. M. WIDDOWSON 1937 Absorption and excretion of iron. Lancet, *2*; 680.

MACY, I. G. 1946 Nutrition and Chemical Growth in Childhood. *2*; 1406, C. C. Thomas, Springfield.

MILLER, L. L., AND P. F. HAHN 1940 The appearance of radioactive iron as hemoglobin in the red cell. J. Biol. Chem., *134*; 585.

MOORE, C. V., R. DUBACH, V. MINNICH AND H. K. ROBERTS 1944 Absorption of ferrous and ferric radioactive iron by human subjects and by dogs. J. Clin. Invest., *23*; 755.

OLDHAM, H., L. J. ROBERTS AND M. YOUNG 1945 Results of providing a liberally adequate diet to children in an institution. III. Blood and urinary excretion studies before and after dietary improvement. J. Pediat., *27*; 418.

PETT, L. B., AND F. W. HANLEY 1946 Hemoglobin Levels of Children in British Columbia. Bull. Vancouver Med. Assoc., *22*; 128.

Rubicon Company Method for total hemoglobin in notes on operation of the Evelyn photoelectric colorimeter, p. 35, Philadelphia.

16th Census of the U.S. 1940 1942 Population and housing statistics for census tracts, Nashville, Tennessee. U. S. Government Printing Office, Washington.

STEINKAMP, R. C., W. D. ROBINSON AND M. M. KASER 1945 Adaptation of short method of calculating the nutritive content of diets in rural areas of Middle Tennessee. J. Am. Diet. Assoc., *21*; 522.

Vanderbilt Cooperative Study of Maternal and Infant Nutrition. To be published.

WINTROBE, M. M. 1933 Macroscopic examination of the blood. Discussion of its value and description of the use of a single instrument for the determination of sedimentation rate, volume of packed red cells, leukocytes and platelets, and of icterus index. Am. J. Med. Sci., *185*; 58.

——— 1942 Clinical Hematology. Lea and Febiger, p. 72, Philadelphia.

THE BIOASSAY OF VITAMIN B_6 IN NATURAL MATERIALS [1]

P. S. SARMA, E. E. SNELL AND C. A. ELVEHJEM
Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

(Received for publication September 30, 1946)

Recently a more nearly synthetic ration for the assay of vitamin B_6 with the rat has been described and the biological activity of pyridoxine, pyridoxamine and pyridoxal determined under various conditions of administration (Sarma, Snell and Elvehjem, '46). Although these 3 compounds were equally active when fed by dropper as daily supplements to the ration, or when injected intraperitoneally, pyridoxal and pyridoxamine were less active than pyridoxine when the 3 compounds were mixed with the ration. This was also true for the chick. Since these 3 compounds occur naturally, and since vitamin B_6 is ordinarily ingested with the ration, it is clear that microbiological assays with Neurospora sitophila (Stokes, Larsen, Woodward and Foster, '43) or Saccharomyces carlsbergensis (Atkin, Schultz, Williams and Frey, '43) for which pyridoxine, pyridoxal and pyridoxamine are equally active (Snell and Rannefeld, '45), do not necessarily reflect the potency of natural foodstuffs as sources of vitamin B_6 for animal nutrition. In the present paper the vitamin B_6 content of several foodstuffs as determined by rat assay on the new

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by a grant from the Wander Company, Chicago, Illinois. We are indebted to Merck and Company, Inc., Rahway, New Jersey, for the generous supply of crystalline B vitamins and to the Abbott Laboratories, North Chicago, Illinois, for the generous supply of haliver oil.

ration is given. The influence of certain dietary factors in modifying the activity of pyridoxal and pyridoxine is also considered. Previous values for the vitamin B_6 content of foodstuffs, as determined by animal assay against a pyridoxine standard, have been reported by Conger and Elvehjem ('41), Henderson, Waisman and Elvehjem ('41), Teply, Strong and Elvehjem ('42).

EXPERIMENTAL

The ration employed for the bioassay of vitamin B_6 and the standard curve obtained with graded amounts of pyridoxine HCl have been described previously (Sarma et al., '46). At least 3 animals were used in each group and in many cases a second group of animals was used in order to confirm the results. With each series of experiments, a curve relating weight gain to concentration of pure pyridoxine HCl was obtained and was found to be similar to that described before. The biological materials to be assayed were mixed in the ration and fed at 2 levels estimated to supply between 25 and 75 µg of vitamin B_6 per 100 gm of ration. Weights were recorded weekly and after an assay period of 4 weeks, the average weekly gain of rats in each group was calculated. The vitamin B_6 content of the material under investigation was obtained by reference to the standard curve in each set of experiments.

A variety of biological materials was assayed in this fashion. The results, expressed as pyridoxine hydrochloride, are summarized in table 1. A number of the same samples was assayed by the yeast growth method of Atkin et al. ('43) in an independent investigation dealing with extraction procedures for vitamin B_6 (Rabinowitz and Snell, '46). These values and other comparative values from the literature are also included in the table.

The vitamin B_6 content of the various materials as determined on the improved ration is of the same order of magnitude as that obtained by other workers. For most of the

foodstuffs the assay has been conducted at 2 different levels. The values obtained at 2 levels, although similar, were never identical. Usually, but not always, higher results were found at the lower level of assay. Similar "drifts" have often been encountered in microbiological assays (Neal and Strong, '43).

TABLE 1

Vitamin B_6 content ($\mu g/gm$) of biological materials calculated as pyridoxine hydrochloride.

MATERIAL	LOWER LEVEL	HIGHER LEVEL	AVERAGE FIGURE	YEAST ASSAY[1]	VALUES IN LITERATURE
Whole liver powder (Wilson)	...	...	10.0	14.2	
Liver powder 1:20 (Wilson)	34.3	31.8	33.1	...	45.0[2], 41.8[3]
Liver fraction L (Wilson)	15.0	14.0	14.5	21.4	
Brewer's yeast	26.0	23.3	24.7	29.5	
Fleischmann's yeast	40.8	37.8	39.3	44.5	55.0[2], 39.3[3]
Duplicate det'n.	43.0	36.0	39.5		
Lamb leg	...	...	4.5	4.6	3.0[4]
Pork ham	6.3	5.2	5.8	...	5.9[4]
Beef liver	...	...	8.1	...	7.3[4], 7.1[3]
Beef kidney	9.2	11.2	10.2	9.9	4.4[4]
Wheat germ	11.6	9.0	10.3	11.2	15.9[4], 9.6[5], 18.0[2]
Whole wheat	2.4	2.9	2.7	4.1	4.1[5], 4.8[3], 4.6[6]
Rolled oats	...	...	2.5	1.9	
Rye	...	...	3.7	...	4.3[6]
Yellow corn	4.5	4.0	4.3	5.7	4.8[4]
Corn grits	...	...	2.5	...	
Raw milled rice	...	...	1.5		
Soybean flour	7.5	6.7	7.1	8.0	
Split peas	...	...	4.0	1.9	
Vitab	...	...	51.7[7]	83.8[7]	
Cerophyl	6.3	6.7	6.5	11.2	8.0[2]
Skim milk powder	...	...	3.8	...	5.5[3]

[1] Rabinowitz and Snell ('46).
[2] Conger and Elvehjem ('41).
[3] Atkin, Schultz, Williams and Frey ('43).
[4] Henderson, Waisman and Elvehjem ('41).
[5] Teply, Strong and Elvehjem ('42).
[6] Stokes, Larsen, Woodward and Foster ('43).
[7] The samples assayed by yeast and rat growth methods were not identical in this case.

That the same phenomenon occurs in animal assays has rarely been pointed out, due chiefly to the fact that assays at more than a single level are not always carried out with animals. In the case of a sample of yeast [2] the experiment was repeated for a second time at 2 different levels when similar variation in values was obtained. For many materials the rat assay values were lower than those obtained on the same samples by the yeast method. These lower values obtained by animal assay are to be expected from the observations made before (Sarma et al., '46) that pyridoxal and pyridoxamine when mixed in the ration were less active than pyridoxine, while for yeast each of the 3 compounds possessed equal activity (Rabinowitz and Snell, '46). Snell ('45) found by microbiological assay using different organisms that in yeast, liver extracts and grass juice, most of the active material is in the pyridoxamine fraction. With each of these substances, rat assay gives lower values than does yeast assay. With some other similar materials, however, (e.g., beef kidney, wheat germ), almost identical values are obtained by the two methods. Determinations of the relative distribution of pyridoxal, pyridoxamine and pyridoxine in these materials are not available.

Influence of dietary factors in modifying the activity of pyridoxal and pyridoxine

In view of the reports in the literature that vitamin B_6 is concerned with metabolism of protein (McHenry and Gavin, '41), particularly that of tryptophane (Miller and Baumann, '45) the effect of additions of various individual compounds, mostly amino acids, on the growth promoting powers of suboptimal quantities of pyridoxine and pyridoxal was studied.

Male weanling rats, after a depletion period of 2 weeks on the modified vitamin B_6-deficient diet (Sarma et al., '46) were fed the experimental diets described in table 2. All the diets contained sucrose as the carbohydrate but the protein component differed in the various diets as indicated in the table.

[2] Fleischmann's yeast, R. C. No. 6.

The average weekly gain represents the average increase in weight during the experimental period of 4 weeks.

The addition of dl-methionine at 1% level decreased the growth gain of vitamin B_6-deficient rats from 5 gm per week to only 1 gm per week (groups 1 and 2). A similar growth

TABLE 2

Influence of certain factors in modifying the activity of pyridoxal and pyridoxine in vitamin B_6 deficient rats.

		AVERAGE WEEKLY GAIN AND RANGE
		gm
1.	Vitamin B_6 deficient diet (18% fibrin)	5 (5–6)
2.	Same as 1 + 1% dl-methionine	1 (1–2)
3.	Vitamin B_6 deficient diet (18% casein) + 50 μg/100 gm of pyridoxine HCl	18 (14–23)
4.	Same as 3 + 1% dl-methionine	6 (3–10)
5.	Same as 3 + 0.5% dl-tryptophane	14 (11–16)
6.	Same as 3 + 1% dl-tryptophane	11 (9–12)
7.	Same as 3 + 0.5% dl-tryptophane + 0.5% indole	6 (2–10)
8.	Same as 3 + 1% l-cystine	16 (15–16)
9.	Vitamin B_6 deficient diet (18% casein + 50 μg/100 gm pyridoxal HCl	11 (7–13)
10.	Same as 9 + 1% dl-methionine	3 (2–4)
11.	Same as 9 + 0.5% indole	8 (6–9)
12.	Same as 9 + 0.5% indole + 0.5% tryptophane	3 (2–5)
13.	Same as 9 + 1% l-cystine	12 (9–16)
14.	Same as 9 + 1% dl-alanine	10 (7–12)
15.	Vitamin B_6 deficient diet (18% fibrin) + 50 μg/100 gm pyridoxine HCl	19 (14–24)
16.	Same as 15 + 5% oleic acid	12 (10–15)
17.	Vitamin B_6 deficient diet (18% fibrin) + 50 μg/100 gm pyridoxal HCl	13 (12–15)
18.	Same as 17 + 5% oleic acid	8 (5–11)

depression was observed when dl-methionine or dl-tryptophane or indole were added to rations containing suboptimal amounts of pyridoxine (groups 3 through 7) or pyridoxal (groups 9 through 12). l-Cystine and dl-alanine did not have any significant effect on the activity of pyridoxal or pyridoxine (groups 8, 13 and 14). The addition of oleic acid at a 5% level to rations containing 50 μg or pyridoxine HCl or pyridoxal

HCl per 100 gm of ration resulted in a growth depression (groups 15 through 18). In all cases where growth inhibition was observed, the addition of 250 µg of pyridoxine HCl or pyridoxal HCl per 100 gm of ration counteracted the growth retardation.

DISCUSSION

Miller and Baumann ('43) in studying xanthurenic acid excretion in mice, found that mice on a diet containing 60% casein required 3 times as much pyridoxine as those on 20% casein diet to reduce the excretion of the pigment to the same level. They also found that the addition of l-tryptophane decreased the survival time of mice deficient in pyridoxine but not to the same extent as the addition of casein of equivalent tryptophane content. From these observations it was clear that amino acids other than tryptophane contributed to the ill health of the pyridoxine-deficient mice. However, additions of tyrosine, histidine and phenylalanine to the diet did not affect the growth or survival time of the deficient mice. The experiments reported here on rats confirm the above observations regarding tryptophane in that additions of tryptophane to the diet containing suboptimal amounts of either pyridoxal or pyridoxine resulted in growth retardation. The experiments also suggest that indole acts in the same manner as tryptophane and that dl-methionine is another amino acid whose metabolism may be intimately connected with the function of vitamin B_6.

Several reports have appeared dealing with the interrelationship between vitamin B_6 and unsaturated fatty acids though the exact mechanism is not clearly understood. Birch ('38) showed that vitamin B_6 and unsaturated fatty acids are both involved in acrodynia, while Schneider, Steenbock and Platz ('40) suggested that essential fatty acids, vitamin B_6 and at least 1 other factor were concerned in the cure of the rat dermatitis. Salmon ('41) and Quackenbush, Kummerow, Platz and Steenbock ('42) have reported similar results. The

growth inhibition obtained with oleic acid and the subsequent counteraction with adequate amounts of vitamin B_6 offers additional evidence that vitamin B_6 may be involved in the metabolism of unsaturated fatty acids.

It is quite unlikely that in the estimation of vitamin B_6, tryptophane, methionine or oleic acid will be present in such high concentration as to interfere with the assay for vitamin B_6. However, the possibility cannot be completely ruled out especially with substances of high protein or fat content and low amounts of vitamin B_6. In such cases suitable controls will have to be devised before the estimation of vitamin B_6 can be satisfactorily carried out. It was previously shown (Sarma et al., '46) that in the presence of large amounts of dextrin, rats grew on the ration without added vitamin B_6. This result was attributed to intestinal synthesis of vitamin B_6 under these conditions. It is possible that with substances low in vitamin B_6, but high in starchy materials, influences of this kind might produce erroneously high values.

SUMMARY

Vitamin B_6 in various biological materials was determined with rats, using the modified vitamin B_6-deficient ration (Sarma et al., '46) and the results compared with the values obtained by other methods. The values obtained are in most cases somewhat lower than the values obtained by the yeast growth method, presumably due to the decreased activity of pyridoxamine and pyridoxal when mixed in the ration.

dl-Tryptophane, indole, dl-methionine, and oleic acid have been shown to exert a growth retarding effect when added to rations containing suboptimal amounts of pyridoxal or pyridoxine. This growth inhibition, however, was counteracted by adequate amounts of the vitamin. l-Cystine and dl-alanine were shown to exert no appreciable effect under similar conditions. The possibility of these dietary factors influencing vitamin B_6 estimation is discussed.

LITERATURE CITED

ATKIN, L. A., S. SCHULTZ, W. L. WILLIAMS AND C. N. FREY 1943 Yeast microbiological methods for determination of vitamins — pyridoxine. Ind. and Eng. Chem., Anal. Ed., *15:* 141.

BIRCH, T. W. 1938 The relation between vitamin B_6 and the unsaturated fatty acid factor. J. Biol. Chem., *124:* 775.

CONGER, T. W., AND C. A. ELVEHJEM 1941 The biological estimation of pyridoxine (vitamin B_6). J. Biol. Chem., *138:* 555.

HENDERSON, L. M., H. A. WAISMAN AND C. A. ELVEHJEM 1941 The distribution of pyridoxine (vitamin B_6) in meat and meat products. J. Nutrition, *21:* 589.

MCHENRY, E. W., AND J. GAVIN 1941 The B vitamins and fat metabolism IV. The synthesis of fat from protein. J. Biol. Chem., *138:* 471.

MILLER, E. C., AND C. A. BAUMANN 1945 Relative effects of casein and tryptophane on the health and xanthurenic acid excretion of pyridoxine-deficient mice. J. Biol. Chem., *157:* 551.

NEAL, A. L., AND F. M. STRONG 1943 Microbiological determination of pantothenic acid. Further studies. Ind. and Eng. Chem., Anal. Ed., *15:* 654.

QUACKENBUSH, F. W., F. A. KUMMEROW, B. R. PLATZ AND H. STEENBOCK 1942 Linoleic acid, pyridoxine and pantothenic acid in rat dermatitis. J. Nutrition, vol. 24, p. 225.

RABINOWITZ, J. C., AND E. E. SNELL 1946 The vitamin B_6 group X. Extraction procedures for the microbiological determination of vitamin B_6. Ind. and Eng. Chem., Anal. Ed., in press.

SALMON, W. D. 1941 The relation of pantothenic acid, pyridoxine and linoleic acid to the cure of rat acrodynia. J. Biol. Chem., *140:* CIX.

SARMA, P. S., E. E. SNELL AND C. A. ELVEHJEM 1946 The vitamin B_6 group VIII. Biological assay of pyridoxal, pyridoxamine and pyridoxine. J. Biol. Chem., *165:* 55.

SCHNEIDER, H., H. STEENBOCK AND B. R. PLATZ 1940 Essential fatty acids, vitamin B_6 and other factors in the cure of rat acrodynia. J. Biol. Chem., *132:* 539.

SNELL, E. E. 1945 The vitamin B_6 group IV. Evidence for the occurrence of pyridoxamine and pyridoxal in natural products. J. Biol. Chem., *157:* 491.

SNELL, E. E., AND A. L. RANNEFELD 1945 The vitamin B_6 group III. The vitamin activity of pyridoxal and pyridoxamine for various organisms. J. Biol. Chem., *157:* 475.

STOKES, J. L., A. LARSEN, C. R. WOODWARD AND J. W. FOSTER 1943 A neurospora assay for pyridoxine. J. Biol. Chem., *150:* 17.

TEPLY, L. J., F. M. STRONG AND C. A. ELVEHJEM 1942 Nicotinic acid, pantothenic acid and pyridoxine in wheat and wheat products. J. Nutrition, *24:* 167.

GROWTH STUDIES WITH RATS KEPT UNDER CONDITIONS WHICH PREVENT COPROPHAGY [1]

ROBERT P. GEYER, BEVERLY RANSONE GEYER, PHILIP H. DERSE, TAYA ZINKIN, C. A. ELVEHJEM AND E. B. HART

Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

TWO FIGURES

(Received for publication October 7, 1946)

In present day nutritional studies involving rats it is possible to prepare highly purified rations of known composition and to select animals from well standardized strains. In this manner some of the more troublesome variables of earlier work are successfully avoided. However, there remain certain factors in all such experimentation which are not so easily controlled. Probably the most important of these is the influence of the intestinal flora, which is free to operate in 2 distinct ways. First, in a direct manner where the products of bacterial synthesis or degradation are absorbed through the intestinal wall of the rat; and second, in an indirect manner where the feces or urine are ingested by the rat. Since both feces and urine contain products of the animal's own metabolism as well as substances of bacterial origin, it is obvious that the animal's own secretions and excretions are a

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by funds granted by the National Dairy Council, Chicago, on behalf of the American Dairy Association; also by funds granted by the Evaporated Milk Association, Chicago. We are indebted to Merck and Co., Inc., Rahway, New Jersey, for supplies of some of the synthetic B vitamins and α-tocopherol; to the Winthrop Chemical Co., Inc., New York, for crystalline vitamin D_2; and to the Wilson Laboratories, Chicago, for the liver powder.

source of variation in nutritional studies. No matter how pure the ration may be or how clean the ordinary type of cage is kept, there always remains the possibility that the animals can obtain contaminating natural products in the form of feces, urine, skin secretions, and hair.

To control the direct effects of the intestinal bacteria, it is necessary to effect a change of some sort in the flora itself. This can be done by the use of bacteria-free animals, or by the easier practice of feeding substances which either alter the kind and strain of bacteria present or else react with compounds produced by the flora. Thus, by the feeding of sulfa-drugs it can be shown that the rat requires a dietary source of biotin (Nielsen and Elvehjem, '42), folic acid (Daft and Sebrell, '43), and vitamin K (Black et al., '42). By the feeding of avidin the rat can be shown to require dietary biotin (Eakin et al., '40).

The control of the indirect influence of the bacterial flora and the animal's own secretions can best be exercised by preventing the animal from eating its feces and urine, and from licking its hair coat. Osborne and Mendel ('11) made the observation that rats allowed to eat their feces grew better than those not permitted to do so. Steenbock et al. ('23) reported that animals receiving feces and half the required amount of vitamin "B" grew much better than those given no feces. That rats would eat 44 to 100% of their feces offered in the dried state was reported by Roscoe ('31), who also showed that rats lived longer on vitamin "B"-free rations when allowed to eat their feces. The work of Dutcher and Francis ('23) on refection caused by feeding raw starch clearly indicated the importance of coprophagy. Though it would appear that the practice of using raised screen floor cages should eliminate coprophagy, one frequently sees animals kept under such conditions obtain feces pellets as they leave the body. It is also very common to see these animals lick themselves and their cages after urination. These observations have led various investigators to attempt the construction of devices designed to prevent coprophagy.

The restriction of the rat's movements by means of a harness has been unsuccessful to date because such mechanisms must be held tightly in place but not so rigidly as to annoy the animal. Furthermore, the harness method of confinement does not prevent the animal from going around the cage and licking off the adhering fecal, urinary, and epidermal residues. To surmount these difficulties investigators have tried to construct coprophagy-preventing cages.

Schwartzer ('37) constructed a cage which was in effect a stationary harness. It consisted of a large sized test tube rack with enlarged holes and which was turned on its side when in use. The animal was so placed that the head, neck, and forepaws passed through the hole, and adhesive tape was used to keep the rat in this position. In these cages weanling rats were reported to survive for an average of 10 days when given a diet of raw whole milk. These rats died before developing anemia, and on autopsy it was found that the gastrointestinal tract was bloody. Comparable animals kept under these conditions but fed their own feces and urine in addition to the milk survived an average of 20 days at which time they showed a hypochromic anemia, but no bloody intestinal tract. No minerals were added to the milk.

Mannering and Cannon ('44) constructed a cage which consisted of an endless circular runway formed by the space between a screen wire bottom and a sheet metal cover which were separated by 2 perpendicular metal bands of unequal diameter. These workers reported no studies in which this cage was used.

Geyer and Boutwell ('45) developed a tubular cage for metabolism studies to prevent any contamination of the excreta by the food. A tube of wire mesh just large enough to hold the rat was attached to a small wooden shaft. A food cup and water bottle were provided. Animals about 200 gm in weight were used and the collection periods were of short duration. Although a number of results were obtained, no growth studies were attempted.

The purpose of this paper is to describe several models of coprophagy-preventing cages and to report some of the nutritional experiments performed with them.

EXPERIMENTAL

Two types of coprophagy-preventing cages were constructed. One was a circular type which allowed the animal an endless circular runway. The other was a tubular type which held the animal in a somewhat fixed position. A detailed description of each and the nutritional studies made with each follow.

The circular type cage

The circular type of cage (fig. 1) consisted of a raised floor (A) made of a disc of metal screening with vertical sheet metal sides (B). One of 2 adjustable metal bands ($\frac{3}{4}$ or $1\frac{1}{2}$

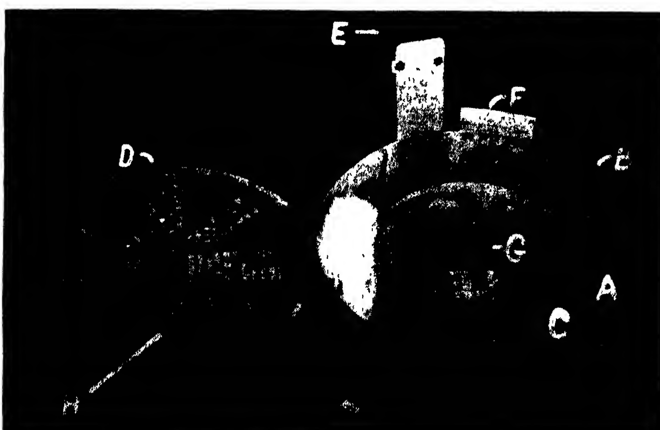


Fig. 1 Circular type anti-coprophagy cage.

inches in height) formed the inner boundary circle (C) and was held in place by a circular screen top (D) which could be raised or lowered to fit the rat. Because of the adjustable width and vertical height, rats varying from 40 gm to 200 gm could be accomodated easily. A water bottle (E) and removable food cup (F) were provided. The rat was placed in

the circular runway and the inner band was enlarged until the animal had a minimum amount of room. The cover was then placed on top and fastened securely by means of the screw adjustment (G). Bolts (H) protruded through the spaces provided in the cover, and by means of the wing-nuts provided the band was held in place. Further adjustments were made as warranted by the growth of the confined animal.

TABLE 1

Experimental rations.

	BASAL RATION I	BASAL RATION II
Sucrose	76	
Lactose or sucrose		48
Casein [1]	18	20
Salts IV [2]	4	4
Corn oil [3]	2	.
Corn oil or butter fat		28

Vitamins added per 100 gm ration:

Diet I: 300 µg thiamine; 400 µg riboflavin; 350 µg pyridoxine; 300 µg nicotinic acid; 200 µg 2-Me-1, 4-naphthoquinone; 7 µg biotin; 2.5 mg Ca pantothenate; and 100 mg choline chloride.

Diet II: Same as for Diet I except for the following: 150 mg choline chloride; 210 µg 2-Me-1, 4-naphthoquinone; 14 µg calciferol [4]; 560 µg beta-carotene [5]; and 2.24 mg alpha-tocopherol.

[1] Extracted for three 2-hour periods with boiling alcohol.

[2] Hegsted et al. ('41).

[3] Mazola brand. Corn Products Refining Co.

[4] Crystalline irradiated ergosterol.

[5] 90% β-carotene and 10% α-carotene.

Twenty-one-day old male rats of the Sprague-Dawley strain weighing approximately 42 gm were used in all of the nutritional studies. Two groups of 3 rats each were placed on experiment in these cages and 2 identical control groups were kept in the usual screen floored dormitory type cages. All feeding was ad libitum. Three rats in each type of cage received the Basal Ration I given in table 1. Each animal received 3 mg of alpha-tocopherol per week in 2 drops of a

1:6 dilution of haliver oil in corn oil. Three rats in each type of cage received the same basal ration plus 2% solubilized liver [2] added at the expense of the entire ration (see table 2). The animals were weighed at weekly intervals and hemoglobin values and both differential and total white cell counts were made at intervals. The data obtained are shown in table 2.

TABLE 2

Results of the experiments with the circular cages. (Each figure represents the average of 3 male rats).

CAGE	RATION	GAIN IN 6 WKS.	HEMOGLOBIN	TOTAL WHITE CELLS	DIFFERENTIAL COUNT
		gm	*(gm/100 ml)*		
Circular	Basal no. I	106	13.38–14.08	7,000– 9,400	Lymph. 75 Neutr. 25
	Basal no. I + S.L.P.[1]	189	13.77–14.08	13,800–17,200	Lymph. 78 Neutr. 20 Monocyte 2
Square — "dormitory type"	Basal no. I	167	12.91–14.28	9,000–15,600	Lymph. 76 Neutr. 24
	Basal no. I + S.L.P.	210	12.91–14.08	13,800–16,000	Lymph. 80 Neutr. 20

[1] Wilson solubilized liver powder (Fraction "L").

The tubular type cage

Two different types of tubular cages were constructed. The first consisted of a cylinder ½-inch mesh wire screen attached to screen wire supports which enabled the hanging of the cage from overhead rods. The cylinder itself was a tube whose longitudinal edges overlapped to allow for expansion as needed. A food cup was held at the front end of the cage by means of a loop of screen wire, and the animal was kept from backing out of the cage by means of a U-shaped wire pin. A water bottle was slung from a wire loop. After several experiments were conducted in this type of cage, several

[2] Wilson's Liver Fraction "L."

mechanical difficulties were found which made construction of another type desirable.

The second type of tubular cage had as its basis the spiral of wire screening with the edges which overlapped covered with strips of metal to prevent injury to the rat. The spiral (A) was attached to a sheet metal support (B) which was cut from one piece of metal in the design shown in figure 2. The food cup (C) slipped into the metal holder (D) in front of the spiral and was held there by tension. When used with weanling rats, a Gooch crucible was placed in the food cup opening

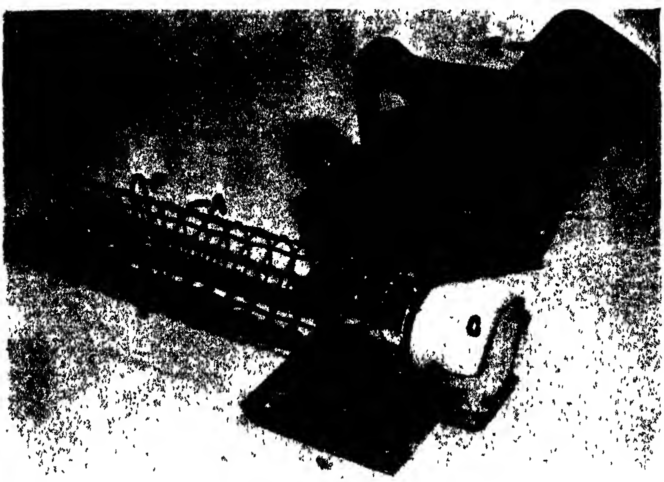


Fig. 2 Tubular type anti-coprophagy cage.

to prevent the small animal from living in the food cup. Such a crucible was found to hold enough food for daily feedings up to the end of the first week of the experiment. The water bottle (E) was held in such a position as to prevent the rat's fur from rubbing on its open end and causing leakage which was found in earlier experiments to affect growth. A U-shaped pin (F) served to keep the animal from backing up. Part of the support was bent to provide a shelf (G) under the front end of the spiral and served to catch the spilled food. The entire cage was suspended from a metal angle iron which

TABLE 3

Results of experiments with the tubular cages.

EXPT. NO.	CARBOHYDRATE	CAGE	FAT		LIVER[1] ADDED	FECES GIVEN	GM[2] GAIN IN 6 WEEKS
			BF[3]	CO[4]			
1	Lactose	Tube	+		−	−	80
				+	−	−	55
			+		+	−	118
				+	+	−	84
		Square dormitory type	+		−	−	142
				+	−	−	140
			+		+	−	146
				+	+	−	136
2	Sucrose	Tube	+		−	−	95
				+	−	−	77
			+		+	−	157
				+	+	−	129
		Square dormitory type	+		−	−	161
				+	−	−	165
			+		+	−	220
				+	+	−	176
3	Sucrose	Tube	+		−	−	121
			+		−	+	104
				+	−	−	77
				+	−	+	81
			+		+	−	155
			+		+	+	153
				+	+	−	124
				+	+	+	123
		Square dormitory type	+		−	−	177
			+		−	+	145
				+	−	−	122
				+	−	+	128
			+		+	−	190
			+		+	+	183
				+	+	−	156
				+	+	+	151

[1] Wilson Fraction "L."

[2] Each figure represents the average of 3 male rats.

[3] Butter fat.

[4] Corn oil.

was large enough to accommodate 4 such cages. To obtain samples of excreta, a low wide dish covered with a metal screen was placed under the rear of the cage.

Weanling male rats of the Sprague-Dawley strain weighing approximately 42 gm were used in these experiments. They were fed the Basal Ration II given in table 1. The carbohydrate portion was either lactose or sucrose, and either butter fat [3] or corn oil was used as the fat. The basal ration was fed with or without the addition of 2% of solubilized liver powder added at the expense of the entire ration. The groups of animals used and the growth responses in 6 weeks are given in table 3. In the second sucrose experiment 3 rats in each group of 6 were fed feces of the animals on the same ration which were housed in the dormitory type cage. Initially 500 mg of wet feces were fed to each animal per day, but the amount was lowered gradually until they were receiving 100 mg per day each. The results of this forced coprophagy are also given in table 3.

RESULTS

When rats were placed in the round cages and fed Basal Ration I, without liver, their growth was sharply curtailed as compared with control rats receiving the same ration but kept in dormitory type cages. As is shown in table 2, when the liver concentrate was incorporated into the ration, the confined animals grew almost as well as the supplemented rats in the square cages. The experiment was repeated with similar results. The rats in the round cages showed normal hemoglobin values but slightly reduced white cell counts.

Rats were kept successfully in the tubular type cages for 6 or more weeks and as shown in table 3, their growth on Basal Ration II containing no liver supplement was poor on either lactose or sucrose. Liver powder was only partially effective in maintaining good growth under these conditions. The animals in the tube cages which received butter fat grew

[3] Prepared from fresh unsalted University Creamery butter by heating at 60–70°C. and decanting from the water and curd.

better on all regimens than the rats fed the corn oil rations. In Experiment 3 the animals fed feces did no better than those fed none. There were a number of animals which showed a watery eye condition and an occasional one that would have tightly closed eye-lids because of the drying of the exudate. Some of the rats developed an eye condition which resembled the "spectacled eye" condition seen in biotin deficiency. All of the animals confined to the tubular cages developed a marked tendency to lick anything within reach of the tongue. Feces feeding did not stop this abnormal behavior.

DISCUSSION

The results obtained with the circular and tubular cages demonstrated that weanling rats will grow under conditions of severe confinement. Their growth was, however, below that of rats fed the same rations but kept in dormitory-type cages. Rats in the latter type of cage grew well with or without liver concentrate in the ration, but the inclusion of the liver gave a definite increase in growth except in the case of the animals fed the lactose diet. In the case of the animals kept in the circular cages very poor growth was obtained without the liver concentrate, whereas, those receiving the liver gained 189 gm in 6 weeks or 83 gm more than those receiving no liver. Since the liver-fed rats in the square cages gained 210 gm during the same period, it seems evident that the importance of feces consumption in the presence of liver is not great. In the absence of liver the eating of feces and other excretory products is of importance since the ration containing no liver allowed 61 gm more gain in 6 weeks for the animals kept in the square cages than the comparable ones confined to the circular cages. Because all of the rations contained biotin, nicotinic acid, and vitamin K, these substances are not responsible for the effect of the feces unless still higher amounts of them are required. Whatever the missing substances are, it is evident that they do not play a role in maintaining either normal hemoglobin or white cell values,

at least under these experimental conditions over a 6-week period.

When animals are kept in the circular cage perfect anti-coprophagy conditions are not attained since the rat travels over screening which it has previously used. Thus, adhering fecal residues are easily licked off and even though the amount is insufficient, it introduces a variable which cannot be controlled. There is also the possibility that urinary and dermal residues are ingested and interfere with obtaining a definite deficiency. In cases where the animals develop diarrhea the circular cage is of little value.

The above difficulties encountered with the circular cage are completely overcome by means of the tubular cage. Whether this fact is reflected in the poorer growth of the rats confined to the tube cages must await further study. Since such animals were unable to get any body excretions except what might be on the forepaws, head, and neck, it would be expected that they would not grow as well in comparison to their controls as did the circular cage animals. Furthermore, the results show that the addition of liver powder to the ration aided the animals, but the growth in no instance was as close to that of the controls as in the circular cage experiment. It must be remembered that in these 2 studies the rations employed were not comparable, especially with respect to the level of fat. At present the effect of various rations under similar experimental conditions is being studied.

The tube cage study demonstrated that the carbohydrate portion of the ration was of importance because where lactose was used the growth was much poorer and the animals in many instances developed severe diarrhea. The growth of the animals in either the tubular or dormitory type cage was below that normally obtained on such diets. On the sucrose rations no diarrhea developed. The carbohydrate present in the studies of Schwartzer ('37) with raw whole milk was, of course, lactose, and this may have added to the poor performance of his rats with or without the feeding of feces. It should be noted that the lactose content of the ration em-

ployed in the work reported in this paper was higher than would be present in whole milk solids, yet the animals grew instead of died. Minerals were not added to the milk employed by Schwartzer, and their absence was probably responsible for the anemia which developed in the animals which survived the longest. It is also possible that the raw milk acted in a manner similar to that of raw starch and that the animals fed feces had the benefit of not only the iron and copper in the feces, but also of some factor or factors needed for survival on such a ration. In the few cases in this laboratory where the effect of the feeding of milk to animals confined in the tubular cages has been tried, the majority of them developed diarrhea even though they were older rats which had been on a milk diet previously to the study.

The data in tabe 3 show that in all 3 experiments the animals confined in the tubular cages grew better on the rations containing butter fat than on the corn oil diets. In the case of the animals kept in the ordinary dormitory cages this difference between fats was inconsistent. It is possible that the anticoprophagy conditions prevent the rats housed in the tubular cages from obtaining certain substances present in feces or urine which have an influence on the nutritive value of the dietary fat. Boutwell et al. ('45) have shown that the B-vitamins in the diet have such an influence.

The tendency of all rats in the tube cages to lick their cages and anything within reach of their tongue is suggestive of a deficiency. To date, the only deficiency in which this curious phenomenon has been reported in the rat is that of potassium (Orent-Keiles and McCollum, '41). It seems unlikely that this element per se is responsible for the condition in the experiments reported here, for it is supplied in liberal amounts in the salt mixture. The condition of the eyes of many of the animals must at present be regarded only as a symptom of unknown origin and must await further study. Biotin was provided, but it is possible that a higher level was required.

The early observations of Osborne and Mendel ('11), Steenbock et al. ('23), and other workers of the period (Kennedy

and Palmer, '28; McCollum et al., '25) cannot be readily compared with the studies reported in this paper for although these investigators reported the beneficial effects of coprophagy, they were working at a time when little was known of the individual vitamin requirements. Since they probably were dealing with inadequate rations, a large part of the influence of the feces could have been due to such vitamins as thiamine and riboflavin. It seems desirable to study with a complete synthetic ration the effect of keeping rats on sheet metal floored cages in contrast to the raised screening type to determine whether or not the added excreta which animals kept in the former type of cage can obtain, is of any importance.

The report of Schwartzer ('37) was based on an incomplete ration, and he was unsuccessful in obtaining healthy rats. Although his method of coprophagy prevention is simple, it seems less convenient than the tubular cage and especially so where excreta collections are desired.

Further studies are needed to determine what factors are required by the rat confined in either the circular or tubular cages. It is also important to ascertain, if possible, whether the probable decrease in exercise is of any real importance. Once these questions have been answered, the tubular cage would make an excellent metabolism cage, for contamination of the excreta by food or reingestion of materials through coprophagy is prevented.

SUMMARY

Two types of coprophagy preventing cages have been constructed and found to allow fair growth of rats over a 6-week period.

In the circular cage described, animals fed no liver concentrate grew poorly; whereas, those receiving such a supplement grew almost as well as control rats kept in ordinary dormitory cages. The imperfections in such a cage are discussed.

Rats confined in the tubular cages grew poorly without a liver supplement, but even when the latter was furnished, growth did not approach as closely to that of control animals

as was found in circular cage experiments. The animals showed a marked tendency to lick everything within reach; some of them had watery eyes and occasionally an eye condition resembling the spectacled eye of biotin deficiency. In all cases, the animals confined to the tubular cages grew better on rations containing butter fat than on comparable rations containing corn oil.

LITERATURE CITED

BLACK, S., R. S. OVERMAN, C. A. ELVEHJEM AND K. P. LINK 1942 The effect of sulfaguanidine on rat growth and plasma prothrombin. J. Biol. Chem., *145:* 137.

BOUTWELL, R. K., R. P. GEYER, C. A. ELVEHJEM AND E. B. HART 1945 Studies on the interrelation of fats, carbohydrates, and B-vitamins in rat nutrition. Arch. Biochem., *7:* 143.

DAFT, F. S., AND W. H. SEBRELL 1943 The successful treatment of granulocytopenia and leukopenia in rats with crystalline folic acid. Pub. Health Rep., *58:* 1542.

DUTCHER, R. A., AND E. FRANCIS 1923 Vitamin studies X. Feeding technique in vitamin studies. Proc. Soc. Exp. Biol. Med., *21:* 189.

EAKIN, R. E., E. E. SNELL AND R. J. WILLIAMS 1940 A constituent of raw egg white capable of inactivating biotin in vitro. J. Biol. Chem., *136:* 801.

GEYER, R. P., AND R. K. BOUTWELL 1945 Unpublished.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

KENNEDY, C., AND L. S. PALMER 1928 The fundamental food requirements for the growth of the rat. IV. Coprophagy as a factor in the nutrition of the rat. J. Biol. Chem., *76:* 607.

McCOLLUM, E. V., N. SIMMONDS AND J. E. BECKER 1925 Technique in the use of the rat for vitamin B studies. J. Biol. Chem., *63:* 547.

MANNERING, G. J., AND M. D. CANNON 1944 Unpublished.

NIELSEN. E., AND C. A. ELVEHJEM 1942 The growth-promoting effect of folic acid and biotin in rats fed succinylsulfathiazole. J. Biol. Chem., *145:* 713.

ORENT-KEILES, E., AND E. V. McCOLLUM 1941 Potassium in animal nutrition. J. Biol. Chem., *140:* 337.

OSBORNE, T. B., AND L. B. MENDEL 1911 Feeding experiments with isolated food-substances, Part II. Carnegie Institution of Washington Pub. no. 156, pt. 2, p. 59.

ROSCOE, M. H. 1931 CCXXIII. The effects of coprophagy in rats deprived of the vitamin B complex. Biochem. J., *25:* 2056.

SCHWARTZER, K. 1937 Über die Bedeutung der Koprophagie bei kuhlmilchernährten jungen Ratten. Z. für Ges. Exp. Med., 101: 502.

STEENBOCK, H., M. T. SELL AND E. M. NELSON 1923 Vitamin B. I. A modified technique in the use of the rat for determinations of vitamin B. J. Biol. Chem., *55:* 399.

COPPER AND MANGANESE STORAGE IN THE RAT, RABBIT, AND GUINEA PIG

EVELYN J. LORENZEN AND SEDGWICK E. SMITH
New York State College of Home Economics, Cornell University; and the U. S. Plant, Soil and Nutrition Laboratory, Agricultural Research Administration, Ithaca, New York

(Received for publication September 26, 1946)

This study was undertaken to determine the storage of copper and manganese in the liver of 3 species, the rat, rabbit, and guinea pig, at various age levels, namely, newborn, 2 weeks, 3 weeks, 2 months, and adult. Analyses were made on the newborn in order to reveal the extent of placental transmission of copper and manganese, at 2 weeks as an indication of the influence of mother's milk since 2 of the 3 species investigated receive milk as their sole source of nutrients during this period, at 3 weeks, which represents the average weaning age, at 2 months, which approximates the period of sexual maturity, and at adult or full maturity.

Although studies of the storage of copper in the liver at various ages in any species are limited in number and involve few experimental subjects, there seems to be agreement that in most species there is a considerable supply deposited during intrauterine life.

Lindow, Peterson, and Steenbock ('29), in studying the metabolism of rats, observed that, as the animal increased in age, there was a continual storage of copper of about 0.001 mg per day for the first 12 days following birth and about 0.002 mg per day thereafter. They were unable to increase the copper content of the newborn rat by feeding the mother a copper-rich diet, indicating that there was neither increased placental

transmission nor increased transmission of copper through the milk.

Further review of the literature on the copper content of livers of small laboratory animals is given in the discussion.

Manganese is apparently transferred through the placenta if present in the mother's blood (Orent and McCollum, '31). A manganese-rich diet fed during gestation produced a noticeable increase in the manganese content of newborn rats, and postpartum supplementation considerably increased the body content of the element at 21, 70, and 180 days (Skinner, Peterson and Steenbock, '31). Manganese is stored slowly in young receiving only mother's milk.

EXPERIMENTAL

Animals

Regular stock colony animals (Dutch rabbits; Sprague-Dawley rats; U. S. Dept. Agric. family 13 inbred guinea pigs) were used in these experiments. The rats were fed dog pellets ad libitum. The rabbits and guinea pigs were fed a good quality mixed hay and a grain mixture consisting of 50 parts by weight of oats and wheat, ad libitum; they also had access to a salt block containing additional iron and iodine. On analysis, the mixed grain, dog pellets, and hay were found to contain 3.1, 12.9, and 5.1 μg copper per gm dry weight and 41.8, 35.0, and 70.7 μg manganese per gm dry weight, respectively.

Ten animals of each species, with the exception of the newborn rats, were etherized at each of the 5 age levels, and the livers removed and weighed immediately. Where possible, individual livers were used for analysis but in the case of the younger animals it was necessary to pool 2 or more in order to obtain a satisfactory size sample. In the case of the newborn rat, the liver was so small that approximately 30 livers were pooled for each sample; thus there are only 3 samples in this age group, representing approximately 100 animals.

The liver was chosen for analysis because of the general agreement (Bunge, 1892; Lindow, Peterson and Steenbock, '29; Cunningham, '31; and Chou and Adolph, '35) that it is a principal site of storage of both manganese and copper in the animal body.

Methods of analysis

Four-gram samples of fresh liver were placed in covered silica dishes, moistened with 2 ml of a 20% solution of $Mg(NO_3)_2$, and ashed in a muffle furnace at 450°C. for a period of 16–18 hours. No dish was used for more than 5 ashings because of the possibility of adsorption of copper (unpublished data) by etched silica. The samples were treated with 2 ml of a nitric-perchloric acid mixture (20:1 by volume), evaporated to dryness on a hot plate, and re-ignited until a white ash was obtained, which in some cases required a second treatment with nitric-perchloric acid. Each ash sample was extracted with three 5 ml portions of N nitric acid and made up to 25 ml volume. Manganese and copper were determined colorimetrically on aliquots of this extract. All analyses were run in duplicate, and unless they agreed within 2 transmission units, were repeated. Copper was determined by the sodium diethyldithiocarbamate method (Callan and Henderson, '29; McFarland, '32; Eden and Green, '40; and Sandell, '44) and manganese by the periodate method (Skinner and Peterson, '30), with slight modifications. The transmission values were read in a photoelectric colorimeter (5 cm absorption cells) using Corning filters 3389 and 5113 for copper, and 4303 and 3486 for manganese determinations. Excellent agreement was obtained in both methods using 2- and 4-gm samples run simultaneously in quadruplicate. Standard solutions of both elements were added separately to 3-gm samples in quadruplicate before ashing, and recovery values ranged from 99 to 102%.

The data obtained were reduced by analysis of variance and the t test used to determine whether particular differences were significant. Odds greater than 19:1 are accepted as being

significant, and odds greater than 99:1 are considered highly significant.

RESULTS

The copper and manganese contents of the livers are presented in 2 different ways — total amount in the liver and concentration. A very different picture of the storage of these elements is obtained depending upon the method of comparison that is used.

Copper

The mean totals and the mean concentrations of copper in the liver of the 3 species at the various age levels are presented in table 1. An analysis of variance of all the data is summarized in table 3. It will be noted that there are differences in both total amount and concentration of copper in the livers among species and among the age groups. These differences are highly significant. Specific differences which appear most important will be pointed out in the following discussion.

It will be noted in table 1 that the total copper of the livers varied greatly among the newborn guinea pigs, rabbits, and rats. These differences were a function of both size of the livers and the concentration of the elements. In the guinea pigs and the rabbits the total liver copper decreased significantly during the suckling period. Following weaning (3 weeks) the total copper increased progressively to reach the highest level in the adult animals. On the other hand, the total copper in the rat livers increased progressively from birth to the adult stages and showed no decrease during the suckling period. In all 3 species there was a very rapid increase in total copper between the ages of 3 weeks and 2 months. This probably is a reflection of the increased copper intake due to the replacement of the copper-poor milk diet with natural feeds containing more copper.

The concentration of copper in the liver presents a picture considerably different from that given by the total copper in

TABLE 1

Total amount and concentration of copper in the liver of guinea pigs, rabbits and rats at the various age levels (mean values with their standard errors).

AGE GROUP	GUINEA PIGS		RABBITS		RATS	
	Content	Concentration	Content	Concentration	Content	Concentration
	μg/liver	μg/gm [1]	μg/liver	μg/gm	μg/liver	μg/gm
Newborn	161 ± 11.7	67 ± 5.6	47 ± 5.8	37 ± 6.7	4 ± 0.3	58 ± 4.0
2 weeks	80 ± 10.7	26 ± 3.5	58 ± 10.2	21 ± 2.4	28 ± 3.2	62 ± 5.0
3 weeks	110 ± 7.0	21 ± 2.4	35 ± 2.2	12 ± 0.6	27 ± 1.9	20 ± 1.2
2 months	264 ± 31.3	19 ± 1.2	213 ± 44.8	24 ± 3.2	63 ± 2.5	9 ± 0.4
Adult	619 ± 92.1	23 ± 3.5	842 ± 98.1	23 ± 3.6	227 ± 17.9	34 ± 2.9

[1] Dry liver.

TABLE 2

Total amount and concentration of manganese in the liver of guinea pigs, rabbits and rats at the various age levels (mean values with their standard errors).

AGE GROUP	GUINEA PIGS		RABBITS		RATS	
	Content	Concentration	Content	Concentration	Content	Concentration
	μg/liver	μg/gm [1]	μg/liver	μg/gm	μg/liver	μg/gm
Newborn	3.1 ± 0.29	1.3 ± 0.13	3.0 ± 0.22	2.3 ± 0.17	0.1 ± 0.01	1.9 ± 0.18
2 weeks	10.8 ± 0.96	3.5 ± 0.23	9.4 ± 1.04	3.4 ± 0.23	0.7 ± 0.06	1.5 ± 0.17
3 weeks	23.4 ± 2.40	4.1 ± 0.30	8.2 ± 0.45	2.7 ± 0.13	3.4 ± 0.14	2.5 ± 0.14
2 months	60.2 ± 4.67	4.4 ± 0.18	32.6 ± 2.71	4.0 ± 0.32	19.2 ± 1.41	2.6 ± 0.14
Adult	108.7 ± 9.49	4.1 ± 0.45	74.8 ± 10.32	1.8 ± 0.13	18.8 ± 2.53	2.6 ± 0.20

[1] Dry liver.

the liver. The concentration of copper in the livers of the newborn rabbits was significantly less (99:1) than in either the guinea pigs or the rats. The guinea pigs and the rats did not differ significantly. In all 3 species the copper concentration decreased significantly (99:1) following birth to reach a minimum at 3 weeks of age in the rabbits and at 2 months in the rats and guinea pigs. The copper concentration found in the adult animals was considerably less than that observed in the newborn young although, as previously mentioned, the total liver copper was much greater in the adult than in the newborn in all 3 species. The copper concentrations in the adult guinea pigs and rabbits did not differ significantly, but the concentration in the adult rat was significantly higher than either. This difference may be due to the fact that the rat diet contained a higher concentration of the element than did the guinea pig or rabbit diet.

Manganese

The mean totals and mean concentrations of manganese in the livers of the 3 species at various age levels are presented in table 2. As in the case of copper, an analysis of variance of the data showed differences which were highly significant among both species and age groups in total amounts and concentrations of the element in the livers.

As a general observation it will be noted that total amounts and concentrations of manganese in the livers are appreciably less than the respective copper values. All 3 species were born with a relatively low content and concentration of manganese. In all 3 species the total amounts of manganese increased in the livers progressively from birth onwards to reach a maximum at the adult stages in guinea pigs and rabbits, and at 2 months in the rats. The total amount of manganese as well as the total amount of copper increased rapidly between the ages of 3 weeks and 2 months in all 3 species.

The manganese concentration in the livers of the 3 species differed significantly at birth, the rabbits having the highest

TABLE 3

Analyses of variance of concentration and total amount of copper and manganese at various age levels.

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE: Copper Content	Copper Concentration	Manganese Content	Manganese Concentration
Total	141				
Species	2	385,076 [1]	1,145 [1]	11,176 [1]	16 [1]
Age groups	4	1,315,884 [1]	5,722 [1]	21,057 [1]	12 [1]
Species X age	8	213,976 [1]	1,740 [1]	3,798 [1]	7 [1]
Error	127	15,497	120	172	0.5

[1] Significant difference at odds greater than 99 : 1.

TABLE 4

Live animal weight, fresh liver weight, and percentage dry matter of the liver of the 3 species (mean values with their standard errors).

AGE GROUP	LIVE ANIMAL WEIGHT (gm)			FRESH LIVER WEIGHT (gm)			PERCENTAGE DRY MATTER		
	G. pigs	Rabbits	Rats	G. pigs	Rabbits	Rats	G. pigs	Rabbits	Rats
Newborn	81 ± 3.0	44 ± 0.6	6 ± 0.1	5 ± 0.1	2 ± 0.1	0.2 ± 0.01	64 ± 1.2	67 ± 0.9	63 ± 0.9
2 weeks	116 ± 5.4	152 ± 12.3	22 ± 1.2	5 ± 0.1	5 ± 0.5	0.7 ± 0.06	76 ± 0.4	70 ± 0.8	74 ± 0.7
3 weeks	190 ± 17.1	166 ± 20.6	47 ± 1.3	9 ± 0.8	6 ± 1.0	2.0 ± 0.10	75 ± 0.4	76 ± 0.8	74 ± 0.2
2 months	420 ± 24.8	327 ± 25.6	188 ± 9.0	20 ± 1.1	12 ± 1.0	11.0 ± 0.60	74 ± 0.3	73 ± 0.4	74 ± 0.2
Adult	918 ± 40.2	2,367 ± 83.3	261 ± 18.7	38 ± 1.3	61 ± 4.8	10.0 ± 1.10	76 ± 0.5	69 ± 1.6	73 ± 0.4

concentrations. The range of variability of manganese concentrations within and between the species were of a relatively low magnitude as compared to copper as well as to many other minerals reported in the literature. The concentration in the livers of guinea pigs increased progressively following birth to reach a maximum at 3 weeks of age. In the rats the concentration decreased during the period of suckling, but not significantly. It reached the maximum value at 3 weeks of age. The mean concentrations of manganese in the rabbit livers in the various age groups were very variable for unexplained reasons. The mean manganese concentration in the adult rabbit livers was significantly less than in either the guinea pigs or the rats.

Table 4 summarizes the live weights, fresh liver weights, and the percentage of dry matter in the livers of the animals used in this study.

DISCUSSION

The data presented indicate that all 3 species, guinea pigs, rabbits, and rats, are born with a reserve store of copper in the liver which is drawn upon during the suckling period. Although the concentration of copper in the livers of the rats decreased during suckling the total amount increased indicating that the rat receives a substantial amount of copper from the milk of the dam. This observation that the milk of rats is relatively rich in copper is supported by the findings of Cox and Mueller ('37) who found rat milk to contain about 10 times as much copper as cow's or human milk.

In contrast to the observations on copper, the data show that the 3 species studied are born with relatively small amounts of manganese in the livers. In all cases the total amount and concentration of manganese in the livers increased progressively from birth onwards, particularly during the period in which substantial amounts of solid feed were consumed. From these observations it cannot be decided whether the small amounts of manganese in the livers at birth of the 3 species indicate a low reserve of manganese or not. Until

more is known of the manganese requirements of young animals the question must remain unanswered. The concentration of manganese in the livers of the species studied varied relatively little during the life span. This observation apparently also holds true for man, for according to Sheldon ('32), "There is no reserve store of manganese provided during fetal life to tide the infant over the nursing period. The manganese content of the liver rises during fetal life and reaches its maximum during the last 3 months, but the maximum is the normal adult level of about 0.0008% of the dry tissue; and once this has been obtained it apparently remains at this figure throughout life."

Many of the studies of nutrient reserves reported in the literature have been concerned only with the concentration of the nutrient in tissues. It will be noted in this study, as well as others previously reported, that a very different picture may be obtained depending on whether one uses total amount or concentration of the nutrient as the criterion of reserve stores. In some cases a decrease in reserves may be indicated by a study of concentration whereas the total amount of the nutrient may actually be increased. Whether concentration or total amount of a nutrient is used is particularly important in studying various age groups in which a difference in size of the liver is a variable influencing particularly the concentration. It is important that care be used in choosing the proper criterion for the particular study undertaken. It would appear that in most cases both total amount and concentration of the nutrient should be determined. The now classic paper of Lintzel and Radeff ('31) is a good example of the use of concentration and total amount of a nutrient (iron) in evaluating the reserve stores of animals.

A comparison of the values which we have obtained for the copper content of the livers of the various animals is compared with values reported by others in table 5. In general there is good agreement in the trends in concentration although the magnitude of concentration varies somewhat. The latter variation may well have been due to the feeding of diets

differing in copper content. For the most part the copper content of the diets has not been reported. A comparison of values of total liver copper as well as the manganese contents of livers of animals is not feasible due to the lack of information.

TABLE 5

Summary of studies on copper in livers recorded in the literature. All data are in mg/kg of dry matter.

AUTHOR	NEWBORN	1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS	8 WEEKS	ADULT
Guinea pigs							
Adrianoff and Ansbacher ('30)	50						16–20
Cunningham ('31)	149	59.6	30.8				17
	203						
Flinn and Inouye ('29)	28.4						37
Lorenzen and Smith (this report)	67.2		26.0	20.9		18.8	23.4
Rabbits							
Cunningham ('31)	5.5						9–10
Lorenzen and Smith (this report)	37.4		21.0	11.6		24.0	22.8
Rats							
Cunningham ('31)	87.8	23.5	14.5	17.8			10–12
McFarlane et al. ('32)	96–121				29–42		
McHargue ('25)	30.3						18.8
Lindow et al. ('29)	70						11.4
Lorenzen and Smith (this report)	58.5		61.6	19.8		8.6	34.2

SUMMARY

Guinea pigs, rabbits, and rats are born with a reserve store of copper which is drawn upon during the suckling period. In rabbits and guinea pigs both total amount and concentration of copper in the livers decreased during the suckling period. On the other hand, rats showed an increase in total copper in the livers from birth onwards even though the concentration decreased during suckling.

In all 3 species studied, the amount of manganese in the livers at birth is relatively low. Both total amount and concentration of manganese in the livers increased from birth throughout life, although the variation in magnitude of concentration of manganese in the livers was relatively small.

It is emphasized that in studies of nutrient storage in animal tissues the determination of only the concentration of nutrients may be insufficient.

ACKNOWLEDGMENT

The authors are grateful to Dr. G. H. Ellis for helpful suggestions on the chemical methods.

LITERATURE CITED

ADRIANOFF, N., AND S. ANSBACHER 1930 Leber und Kupfer. Deutsche med. Wchnschr, *56:* 357.

BOYER, P. D., J. H. SHAW AND P. H. PHILLIPS 1942 Studies on manganese deficiency in the rat. J. Biol. Chem., *143:* 417.

BUNGE, G. 1892 Weitere Untersuchungen über die Aufnahme des Eisens in den Organismus des Saüglings. Ztschr. f. physiol. Chem., *16:* 173.

CALLAN, T., AND J. A. R. HENDERSON 1929 A new reagent for the colorimetric determination of minute amounts of copper. Analyst, *54:* 650.

CHOU, T., AND W. H. ADOLPH 1935 Copper metabolism in man. Biochem. J., *29:* 476.

COX, W. J., AND A. J. MUELLER 1937 The composition of milk from stock rats and an apparatus for milking small laboratory animals. J. Nutrition, *13:* 249.

CUNNINGHAM, I. J. 1931 Some biochemical and physiological aspects of copper in animal nutrition. Biochem. J., *25:* 1267.

EDEN, A., AND H. H. GREEN 1940 Micro-determination of copper in biological materials. Biochem. J., *34:* 1202.

FLINN, F. B., AND J. M. INOUYE 1929 Some physiological aspects of copper in the organism. J. Biol. Chem., *84*, 101.

LINDOW, C. W., W. H. PETERSON AND H. STEENBOCK 1929 The copper metabolism of the rat. J. Biol. Chem., *84:* 419.

LINTZEL, W., AND T. RADEFF 1931 Über den Eisengehalt und Eisenansatz neugeborener und saugender Tiere (nach Versuchen an Kaninchen, Meerschweinchen, Ratte, Hund, Katze, Schwein, Ziege, Rind). Arch. F. Tierernahrung u. Tierzucht, *6:* 313.

MCFARLANE, W. D. 1932 Application of the sodium diethyldithiocarbamate reaction to the micro-colorimetric determination of copper in organic substances. Biochem. J., *26:* 1022.

McHargue, J. S. 1925 The association of copper with substances containing the fat soluble A vitamin. Am. J. Physiol., *72:* 583.

Orent, E. R., and E. V. McCollum 1931 Effects of deprivation of manganese in the rat. J. Biol. Chem., *92:* 651.

Sandell, E. B. 1944 Colorimetric Determination of Traces of Metals. Interscience Publishers, Inc., N.Y.C.

Sheldon, J. H. 1932 Some considerations on the influence of copper and manganese on the therapeutic activity of iron. British M. J., *ii:* 869.

Skinner, J. T., and W. H. Peterson 1930 The determination of manganese in animal materials. J. Biol. Chem., *88:* 347.

Skinner, J. T., W. H. Peterson and H. Steenbock 1931 The manganese metabolism of the rat. J. Biol. Chem., *90:* 65.

SOME PHYSIOLOGICAL RELATIONSHIPS OF PROTEIN, FAT, CHOLINE, METHIONINE, CYSTINE, NICOTINIC ACID AND TRYPTOPHANE [1]

W. D. SALMON
Laboratory of Animal Nutrition, Agricultural Experiment Station, Alabama Polytechnic Institute, Auburn

(Received for publication August 23, 1946)

In 1915, Osborne and Mendel reported that diets containing 15 to 18% of casein along with essential nonprotein components produced normal growth in young rats; when similar diets containing 9 to 12% of casein were fed, growth was definitely limited but could be significantly increased by the addition of cystine. Jackson and Block ('32) later found that methionine also supplemented low levels of casein. Still later Womack and associates ('37, '41) concluded that methionine could replace cystine entirely and that the latter was effective only in the presence of a certain minimum amount of methionine. These findings have extended rather than questioned the original observations of Osborne and Mendel, which have been confirmed by other investigators.

In work relating to the physiology of choline and the B vitamins in 1941, however, it was observed in this laboratory [2] that growth was decidedly subnormal on low-fat, low-casein diets, and was depressed rather than increased by the addition of cystine. A reinvestigation of the nutritive value of casein was therefore undertaken. The results show some interesting relationships of protein, fat, certain amino acids, and vitamins.

[1] Published with the permission of the director of the Alabama Agricultural Experiment Station.

[2] Unpublished data by the author.

METHODS AND RESULTS

General procedure

A line-bred strain of hooded rats from the Wisconsin original stock was used throughout this investigation. Rats were weaned at 23 days of age and caged individually in metal cages with raised screen bottoms. Fresh tap water and food were supplied daily, ad libitum. Once a week all food residues were weighed and discarded and clean food jars were placed in the cages. The floor screens and the papers in drip pans were changed each week. The rats were weighed weekly at approximately the same time of day. Each group, with 5 exceptions, consisted of 2 males and 2 females, and the groups being compared always represented uniform litter composition. For economy of space the comparable control groups have been combined in tables 3 and 4.

The composition of the various basal diets is given in table 1. Adequate amounts of α-tocopherol, carotene, calciferol, thiamine, riboflavin, pyridoxine, inositol, and Ca pantothenate were mixed into the basal diets. Choline chloride was omitted from the diets used in the experiments listed in table 2 and for certain groups that received methionine as indicated in tables 3 and 4.

Special supplementary additions and major changes of diet are shown in tables 2 to 5.

Primary deficiency of casein is labile methyl

Much work has been done on choline-deficient diets containing various levels of casein. The results have shown that casein has lipotropic and kidney-protecting properties. Griffith's ('41) review gives a bibliography of the important work to 1941; see also Treadwell et al., '42, Beveridge et al., '44, and Handler, '46. Some of the results have been complicated by the inclusion of other proteins in casein diets and by the use of yeast or yeast extracts as sources of vitamins.

In the beginning of this study, rats that received choline-free diets containing crystalline vitamins and low levels of casein

TABLE 1

Composition of basal diets. Major ingredients and percentages thereof.[1]

DIET NUMBER	CASEIN [2]	SALT [3]	CARBOHYDRATE [4]	FAT [5]	CHOLINE [6]
6	6	3	90.8		0.2
8.3 L	8.3	3	58.5	30	0.2
9	9	4	86.8		0.2
12	12	4	83.8		0.2
12 L/2	12	4	68.8	15	0.2
12.5 L	12.5	4	53.3	30	0.2
15	15	4	80.8		0.2
16.5 L	16.5	4	49.3	30	0.2
18	18	4	77.8		0.2
18 L/2	18	4	62.8	15	0.2
18 L	18	4	47.8	30	0.2
24	24	4	71.8		0.2
24 L/2	24	4	56.8	15	0.2
25	25	4	70.8		0.2
25 L	25	5.5	39.3	30	0.2
30	30	4	65.8		0.2
30 L/2	30	4	50.8	15	0.2
41 L	41	5.5	23.3	30	0.2

[1] Additions of special supplements as noted in later tables were always at the expense of the carbohydrate component and were always in addition to the regular vitamin supplements carried by all diets in the following amounts in mg/kg of diet: carotene 4, α-tocopherol 50, calciferol 0.125, i-inositol 200, thiamine 2, pyridoxine 2, riboflavin 4, Ca pantothenate 10. Nicotinic acid was added only in certain groups as indicated in tables 2 and 5. The calciferol was donated by Mead Johnson Co., Evansville, Ind.

[2] The casein was purified in 50-lb. lots by cold percolation with tap water for 6 days. The water standing in the casein overnight was acidified to 0.20% with acetic acid. On the seventh day the acid was washed out and about 4 gal. of alcohol percolated through the casein to facilitate drying under a fan in a warm room.

[3] In the early experiments Salt 186 (J. Biol. Chem., 1930, vol. 89, p. 199) was used. In the later experiments Salt 5 was used. The composition of Salt 5, as mixed, was $Ca_3(PO_4)_2$ 2050, K_2HPO_4 1030, $MgSO_4 \cdot 7H_2O$ 600, NaCl 500, Fe citrate 130, $MnSO_4 \cdot 4H_2O$ 20, $CuSO_4 \cdot 5H_2O$ 5, $ZnCO_3$ 10, KI 5 gm. This was dried at 70°C. to a dry weight of 4000 gm.

[4] Sucrose (standard granulated cane sugar) was the carbohydrate used except for a few groups where it was replaced with glucose (cerelose) or cornstarch as shown in tables 3 and 4.

[5] Lard was the fat used except for 7 groups as shown in table 4. In all cases where the diet did not contain added fat, each rat received 0.10 ml/day of corn oil as a source of essential fat acids.

[6] Choline was omitted and the carbohydrate increased 0.20% for all groups listed in table 2.

died. The studies on the choline-free diets were extended. From the results shown in table 2, it is evident that the primary deficiency of casein at low levels is labile methyl. Kidney lesions and death occurred even at casein levels of 18 and 24% in choline-free diets containing a minimum of fat and no added cystine. At levels of 9, 12, and 15%, kidney lesions and mortality ranged from 75 to 100%. The addition of cystine or

TABLE 2

Mortality rate of rats receiving various levels of casein in choline-free diets.

BASAL DIET	FAT LEVEL	l-CYSTINE ADDED	NUMBER OF RATS	MORTALITY RATE [1]	AV. TIME TO DEATH	KIDNEY LESIONS	BASAL DIET	FAT LEVEL	l-CYSTINE ADDED	NUMBER OF RATS	MORTALITY RATE [1]	AV. TIME TO DEATH	KIDNEY LESIONS
		%		%	days	%			%		%	days	%
9	[3]		8	75	14	100	18	[3]	0.3	4	25	9	25
9	[3]	0.3	8	100	12	100	18	[3]	0.5	4	100	9	100
9 [2]	[3]		8	87.5	14	87.5	18	[3]	1.0	4	100	9	100
9 [2]	[3]	0.3	8	100	11	100	18	[3]	1.5	4	100	12	100
12	[3]		20	100	10	100	18 [1]	[3]	0.3	4	75	10	100
12	[3]	0.3	4	100	9	100	18 L/2 [1]	[4]	0.3	4	75	10	100
12 [2]	[3]	0.3	3	100	9	100	24	[3]		4	25	9	25
12 L/2 [2]	[4]	0.3	11	100	9	100	24	[3]	0.3	4	25	9	37.5
15	[3]		4	75	10	75	24 L/2	[4]	0.3	4	25	9	75
15	[3]	0.3	4	100	10	100	30	[3]	0.3	4	0		0
18	[3]		8	25	9	37.5	30 L/2	[4]	0.3	4	25	10	50

[1] Mortality rate indicates rats dying of hemorrhaged kidneys within the 3-week experimental period. Any survivors on the twenty-first day were killed and examined for kidney lesions.

[2] 20 mg of nicotinic acid was added per kilo of diet for these groups.

[3] 0.10 ml corn oil/rat/day.

[4] 15% pure lard in diet.

cystine and fat tended to increase the incidence of kidney lesions and the rate of mortality at the higher levels of casein and to decrease the survival period at the lower levels.

Mulford and Griffith ('42) have suggested that the harmful effect of cystine in choline-free diets is related to increased rate of growth. In our experiments, however, cystine added to a low-fat, low-casein diet depressed rather than increased the growth rate (table 3).

Although 24% of casein in the low-fat diet did not afford complete protection against kidney lesions, 0.38% of free dl-methionine added to a 12%-casein diet did. When the methionine was supplied, the further addition of choline was

TABLE 3

Gains of rats receiving various levels of casein, cystine, methionine and other supplements in low-fat diets for 8 weeks.

BASAL DIET	DIET CHANGE OR SPECIAL SUPPLEMENT ADDITION	NO ADDED CYSTINE			0.30% ADDED l-CYSTINE		
		Number of rats	Av gain per rat	Gm food /gm gain	Number of rats	Av gain per rat	Gm food /gm gain
			gm			*gm*	
6		4	—1		4	—5	
6	0.38% Methionine	4	—2				
9		8	36	8.20	8	21	10.52
9	0.38% Methionine	4	28	8.60			
9	6% Yeast	4	89	4.48	4	89	4.18
12		24	59	5.44	24	32	7.53
12	0.38% Methionine	12	36	6.33			
12	0.38% Methionine without choline	12	66	4.92			
12	6% Yeast	8	147	4.22	8	157	3.69
12	6% Yeast residue[1]	4	77	5.23	4	70	5.13
12	6% Liver residue[2]	4	94	4.70	4	88	4.43
12	6% ≎ Liver extract[3]	4	108	4.90	4	132	4.13
12	Glucose for sucrose	4	98	5.65	4	69	6.40
12	Cornstarch for sucrose	12	113	4.65	12	96	4.78
15		4	111		4	77	
18		12	115	3.88	12	93	4.13
18	0.38% Methionine	4	116	3.51	4	104	3.48
18	0.38% Methionine without choline	4	126	3.54	4	122	3.60
18	6% Yeast	16	195	3.69	8	182	3.84

[1] The residue from exhaustive extraction of brewer's yeast with 51% (by wt.) alcohol.

[2] The residue from similar extraction of dried beef-liver.

[3] The 51%-alcohol extract from dried beef-liver, concentrated under diminished pressure, and added to diet at level equivalent to 6% of dried liver.

not beneficial (table 3). Likewise, 0.38% dl-methionine in a diet containing 12.5% casein and 30% lard afforded complete protection against kidney lesions in rats that made an average gain of 156 gm in 8 weeks (table 4). In contrast with this, rats

receiving 30% casein, 0.30% cystine and 15% lard showed 50% incidence of kidney damage and 25% mortality. Free methionine was clearly more effective in preventing kidney damage than methionine combined in the casein molecule. This augments the finding of Eckstein and co-workers (Tucker and Eckstein, '37; Tucker, Treadwell and Eckstein, '40; and Treadwell, Groothuis and Eckstein, '42) that the lipotropic action of free methionine was superior to that of methionine combined in casein.

Best and Ridout ('40) and Channon et al. ('43) have not agreed that the lipotropic effect of protein can be explained on the basis of methionine alone. More recent work from Best's laboratory (Beveridge et al., '44) has shown no significant difference in the lipotropic action of free methionine and methionine combined in casein provided that the essential amino acids were approximately equal. One would expect more of the methionine methyl to be available for choline synthesis when methionine is fed as the sole supplement than when it is fed along with other essential amino acids or combined with other essential amino acids in the protein molecule.

Cystine fails to supplement casein in low-fat diets

The growth of rats receiving low-fat diets containing various levels of casein, the usual crystalline vitamins, and choline or methionine as a source of transferable methyl groups, was studied next. The data are summarized in table 3. It is apparent that such diets even at the 18% casein level fall far short of producing normal growth. At the 12 and 9% casein levels growth was only slightly above one-fourth and one-sixth, respectively, of the normal rate.

The addition of cystine not only failed to increase the rate of growth but actually decreased it, particularly at the 9 and 12% levels of casein.

The addition of 6% brewer's yeast produced a remarkable response in growth. The increase was very significant at the 9 and 12% casein levels, and growth approached the normal rate at the 18% level. Liver extract also markedly increased

growth on the 12% casein diet. Moreover, yeast or liver extract counteracted the depressing effect of cystine.

The extracted residues of yeast or liver were relatively ineffective in increasing growth. When sucrose was replaced by cornstarch there was a significant improvement in growth but the rate was still abnormal.

Fat increases growth rate and alters effect of cystine

Since the diets used by Osborne and Mendel had contained substantial amounts of fat, the effect of adding 30% lard to the basal diet was studied. The casein was increased to provide the same ratio between protein and nonprotein calories as in the low-fat diets. The results are presented in table 4.

TABLE 4

Gains of rats receiving various levels of casein, cystine, methionine and other supplements in 30%-fat diets for 8 weeks.

BASAL DIET	DIET CHANGE OR SPECIAL SUPPLEMENT ADDITION	NO ADDED CYSTINE			0.30% ADDED l-CYSTINE		
		Number of rats	Av. gain per rat	Gm food /gm gain	Number of rats	Av. gain per rat	Gm food /gm gain
			gm			*gm*	
8.3 L		3	10	17.30	4	31	6.90
8.3 L	0.38% dl-Methionine	4	40	5.77			
12.5 L		8	76	3.79	8	141	2.66
12.5 L	0.38% dl-Methionine	8	126	2.67			
12.5 L	0.38% dl-Methionine without choline	4	156	2.80			
16.5 L		12	135	2.96	8	171	2.47
16.5 L	0.38% dl-Methionine	4	183	2.28	4	176	2.27
16.5 L	0.38% dl-Methionine without choline	12	186	2.42			
25 L		16	199	2.47	16	225	2.35
25 L	0.45 dl-Methionine	4	207	2.23	4	200	2.10
25 L	0.45 dl-Methionine without choline	4	227	2.10			
25 L	6% Yeast				7	238	2.63
25 L	Cornstarch for sucrose	4	201	2.52	4	196	2.50
25 L	Butterfat for lard	4	204	2.96	4	205	2.46
25 L	Margarine fat for lard	4	204	2.86	4	204	2.49
25 L	Coconut oil for lard	4	189	3.04			
41 L		8	205	2.23	8	214	2.24

The fat addition resulted in marked improvement of growth at all levels of protein. Whereas all rats had lost weight on the low-fat, 6%-casein diet, all gained on the 30%-lard, 8.3%-casein diet. On the 25%-casein diet with 30% lard, the gains were essentially normal and about double the gain on the corresponding 18%-casein, low-fat diets when crystalline vitamins were used as the only supplements.

Another striking effect of the fat addition was that cystine additions resulted in further improvement of growth rate at low levels of casein. Moreover, brewer's yeast, which had effected marked improvement in growth on the low-fat diets, had little effect with the fat diets containing 25% casein.

Butterfat and margarine fat were comparable to lard in stimulating growth. Coconut oil was only slightly less effective; this may have been caused by a lack of essential unsaturated fat acids in the coconut oil as no other source of these dietary factors was supplied with the fat diets. The general effect of fats in increasing the rate of growth, however, was not associated with unsaturated fat acids. The corn oil fed with the low-fat diets furnished an adequate supply of linoleic acid.

Nicotinic acid or tryptophane corrects second deficiency of casein in low-fat diets

After the marked growth-stimulating effect of fat was observed and it was found that the active principle of yeast and liver was water-soluble, increased levels of thiamine, riboflavin, pyridoxine, and Ca pantothenate were tested. The additions of para-amino-benzoic acid, 2-methyl 1, 4-napthoquinone, and of biotin,[3] all of which had previously given negative results in similar diets, were rechecked. The results of these tests were negative. Nicotinic acid also had given negative results with rats in this laboratory in 1939, when added to a black-tongue-producing ration. No convincing evidence of its need by rats had appeared from other labora-

[3] Supplies of biotin were donated by the Medical Department of Merck and Co., Rahway, N. J.

tories. This vitamin fortunately was not included as a routine supplement to the diets in the early part of the investigation. Dann's ('41) report, appearing shortly after the study was initiated, seemed to prove conclusively that dietary nicotinic acid was not needed by the rat.

Since the diet used by Dann contained 25% casein, however, it was decided to try nicotinic acid as a supplement to the low-casein, low-fat diets. The results (table 5) were surprising indeed. When 20 mg of nicotinic acid per kilo of diet was added,

TABLE 5

Effect of nicotinic acid or tryptophane on gains of rats receiving various levels of casein, cystine, and fat for 8 weeks.

BASAL DIET	FAT LEVEL	l-CYSTINE ADDED	NO NICOTINIC ACID			NICOTINIC ACID ADDED		
			Number of rats	Av. gain per rat	Gm food /gm gain	Number of rats	Av. gain per rat	Gm food /gm gain
		%		gm			gm	
9	0.10 ml corn oil/rat/day	0.1	4	22	9.70	4 [1]	63	5.19
12	0.10 ml corn oil/rat/day		4	44	5.89	3 [1]	68	4.69
12	0.10 ml corn oil/rat/day	0.3	8	27	8.07	8 [1]	104	4.22
12	0.10 ml corn oil/rat/day	0.3	4	38	7.37	4	175	4.08
12	0.10 ml corn oil/rat/day	0.3	4 [2]	171	3.70	4 [2]	172	3.83
12	0.10 ml corn oil/rat/day	0.3	4	24	8.41	4	137	3.49
12 L/2	15% lard	0.3	4	62	4.03	4	162	2.84
16.5 L	30% lard	0.3	4	166	2.38	4 [1]	179	2.39
18	0.10 ml corn oil/rat/day	0.3	8	90	4.18	12	180	3.66
18 L	30% lard	0.3	4	198	2.31	4	205	2.26
25	0.10 ml corn oil/rat/day	0.3	4	196	3.73	4	199	3.80
25 L	30% lard	0.3	4	218	2.23	4	227	2.21

[1] The diet for these 4 groups of rats contained 1 mg % of nicotinic acid; the diet for all other rats receiving nicotinic acid contained 2 mg %.

[2] 0.3% dl-tryptophane was added to the diet for these 2 groups.

improvement in growth was as striking as when brewer's yeast or liver extract was added. The effect was greatest at the 12% level of casein but was very significant even at the 18% level. With 25% of casein in the low-fat diet, growth was essentially normal and was not improved materially by nicotinic acid.

Nicotinic acid, moreover, had a similar effect to yeast and fat in permitting cystine to manifest a supplemental action at low levels of casein.

In the diet containing 30% lard and 16.5% casein, which on a protein: calorie basis was comparable to the 12%-casein, low-fat diet, nicotinic acid had a relatively slight effect on growth. When the casein was increased to 25% in the lard diet, which is comparable to the 18% of casein in the low-fat diet, there was no significant effect of nicotinic acid on growth.

In the meantime the Wisconsin workers (Krehl et al., '45) reported that tryptophane had an effect similar to that of nicotinic acid in supplementing diets having a relatively high content of corn products The addition of 0.30% dl-tryptophane to the 12%-casein diet produced about the same growth as the addition of 20 mg of nicotinic acid per kilo of diet. There was no apparent advantage to the addition of both tryptophane and nicotinic acid. These results showed definitely that the second limiting factor in the low-fat diets containing 18% or less of casein was nicotinic acid or tryptophane. Since the completion of these experiments, Krehl et al. ('46) have reported that the relationship between nicotinic acid and tryptophane is not peculiar to corn-supplemented rations but is related to the nature of the total amino acid content of the ration.

Results emphasize protein, fat, amino acid, and vitamin relationships

The results reported in this paper illustrate how the apparent nutritive value of a protein may be influenced by other dietary constituents. First in importance is the effect of

transferable methyl groups. In the absence of choline from the diet, labile-methyl groups become the first limiting factor of casein. For strains of rats having a relatively high choline requirement (Engel, '43; Copeland, '44), this limitation is disastrous in diets containing 15% or less of casein. It is significant even at levels of 24 to 30% casein, particularly if the diets contain cystine and fat.

The effect of nicotinic acid is also of major importance. It appears that there is maximum need for nicotinic acid when the diet is high in sugar and low in fat. In the absence of adequate nicotinic acid, the rat may be able to convert tryptophane to nicotinic acid to supply the metabolic requirements for this vitamin. This view is supported by the results of Rosen, Huff and Perlzweig ('46) and of Singal et al. ('46), indicating that tryptophane may be the precursor of nicotinic acid synthesis in the rat. Obviously in the low-casein diets the tryptophane supply is not adequate for this purpose and for tissue formation at the normal rate. The relatively high level of 25% casein is required to furnish sufficient tryptophane to build tissues and meet the nicotinic acid requirements of the growing rat on a nicotinic acid-free, low-fat diet. Wintrobe et al. ('45) have recently reported that pigs showed marked symptoms of nicotinic acid deficiency on a 10%-casein ration but not on a 26.1%-casein ration.

The marked growth-promoting effect of fat in the nicotinic acid-free, low-casein diets is explained on the basis of a nicotinic acid-sparing action of fat. The relatively high levels of fat required are in line with such an interpretation. The effect is nearly as striking and may be as important nutritionally as the thiamine-sparing action of fat. The higher energy value of fat diets and the possible economy of utilization of preformed fat as compared with the synthesis of fat from carbohydrate may be of some importance. The excellent growth that results when nicotinic acid is added to the low-fat diets, however, indicates that these are factors of minor importance. These results can be explained on the basis that nicotinic acid, like thiamine, functions primarily in carbohy-

drate metabolism. They also provide further information regarding the intrinsic importance of dietary fat.

A partial explanation of the anomalous effect of cystine in the low-fat, low-casein diets now becomes obvious. Cystine added to such diets cannot be utilized because of the deficiency of nicotinic acid or tryptophane. Only when this deficiency is remedied, is a deficiency of cystine demonstrable. It is not clear why the addition of cystine to the nicotinic acid-free, low-casein, low-fat diets should be harmful.

The low-casein diets used by Osborne and Mendel ('15) contained 28% protein-free milk, 25% fat, and 18 to 30% starch. These ingredients probably remedied the deficiencies of labile methyl and nicotinic acid in the casein. Under such conditions cystine (or sulfur containing amino acid) was the factor limiting growth at the 9 and 12% levels of casein.

Although methionine can be used as the source of labile methyl and tryptophane or casein at high levels as the source of nicotinic acid (certainly for rats and probably for pigs), the economy of using choline and nicotinic acid is obvious. The presence of these 2 vitamins increases the efficiency of protein utilization and thus makes relatively low-protein diets adequate for growth. That the nicotinic acid-sparing action of fat may also play an important role in practical nutrition is clearly indicated. In decreasing the need for nicotinic acid, fat may indirectly conserve ingested tryptophane and thus increase the efficiency of protein utilization. The protein required per gm of gain was significantly less in the animals receiving 30% fat than in those receiving the low-fat diets.

SUMMARY

1. The primary deficiency in diets containing 18% or less of casein was found to be labile-methyl groups unless supplementary choline or methionine was added.

2. The deficiency of labile methyl was aggravated by the addition of cystine or cystine and fat.

3. Supplementary methionine as the free amino acid was more effective than methionine combined in the casein molecule.

4. The second demonstrable deficiency in low-fat diets containing 18% or less of casein was nicotinic acid.

5. Tryptophane or a high level of casein (25%) counteracted the deficiency of nicotinic acid.

6. A high level of fat (30%) tended to counteract the deficiency of nicotinic acid. This was probably a nicotinic acid-sparing effect as the energy metabolism shifted from carbohydrate to fat and indicates that nicotinic acid, like thiamine, functions primarily in carbohydrate metabolism.

7. Only when the deficiencies of labile methyl and nicotinic acid were remedied, was a deficiency of cystine (or sulfur amino acid) demonstrable.

8. Methionine corrected both the labile methyl and the sulfur amino-acid deficiencies.

9. The possible nutritional significance of the findings is discussed.

LITERATURE CITED

Best, C. H., and J. H. Ridout 1940 The lipotropic action of methionine. J. Physiol., *97:* 489.

Beveridge, J. M. R., C. C. Lucas and M. K. O'Grady 1944 The effect of the nature and level of protein and amino acid intake upon the accumulation of fat in the liver. J. Biol. Chem., *154:* 9.

Channon, H. J., G. T. Mills and A. P. Platt 1943 The action of amino acids and proteins on liver-fat deposition. Biochem. J., *37:* 483.

Copeland, D. H. 1944 Strain differences in the choline requirement of rats. Proc. Soc. Exp. Biol. Med., *57:* 33.

Dann, W. J. 1941 The synthesis of nicotinic acid by the rat. J. Biol. Chem., *141:* 803.

Engel, R. W. 1943 Inherited differences in the choline requirement of rats. Proc. Soc. Exp. Biol. Med., *52:* 281.

Griffith, W. H. 1941 The nutritional importance of choline. J. Nutrition, *22:* 239.

Handler, Philip 1946 Factors affecting the occurrence of hemorrhagic kidneys due to choline deficiency. J. Nutrition, *31:* 621.

Jackson, R. W., and R. J. Block 1932 Metabolism of cystine and methionine. The availability of methionine in supplementing a diet deficient in cystine. J. Biol. Chem., *98:* 465.

KREHL, W. A., P. S. SARMA AND C. A. ELVEHJEM 1946 The effect of protein on the nicotinic acid and tryptophane requirements of the growing rat. J. Biol. Chem., *162:* 403.

KREHL, W. A., L. J. TEPLY, P. S. SARMA AND C. A. ELVEHJEM 1945 Growth-retarding effect of corn in nicotinic acid-low rations and its counteraction by tryptophane. Sci., *101:* 489.

MULFORD, D. J., AND W. H. GRIFFITH 1942 Choline metabolism. VIII. The relation of cystine and methionine to the requirement of choline in young rats. J. Nutrition, *23:* 91.

OSBORNE, T. B., AND L. B. MENDEL 1915 The comparative nutritive value of certain proteins in growth and the problem of the protein minimum. J. Biol. Chem., *20:* 351.

ROSEN, F., J. W. HUFF AND W. A. PERLZWEIG 1946 The effect of tryptophane on the synthesis of nicotinic acid in the rat. J. Biol. Chem., *163:* 343.

SINGAL, S. A., A. P. BRIGGS, V. P. SYDENSTRICKER AND J. LITTLEJOHN 1946 Effect of tryptophane on urinary excretion of nicotinic acid in rats. Fed. Proc., *5:* 154.

TREADWELL, C. R., M. GROOTHUIS AND H. C. ECKSTEIN 1942 The influence of supplementary casein, cystine, and methionine on liver lipid content. J. Biol. Chem., *142:* 653.

TUCKER, H. F., AND H. C. ECKSTEIN 1937 The effect of supplementary methionine and cystine on the production of fatty livers by diet. J. Biol. Chem., *121:* 479.

TUCKER, H. F., C. R. TREADWELL AND H. C. ECKSTEIN 1940 The effect of supplementary cystine and methionine on the production of fatty livers by rats on high fat diets containing casein or edestin. J. Biol. Chem., *135:* 85.

WINTROBE, M. H., H. J. STEIN, R. H. FOLLIS, JR. AND S. HUMPHREYS 1945 Nicotinic acid and the level of protein intake in the nutrition of the pig. J. Nutrition, *30:* 395.

WOMACK, M., AND W. C. ROSE 1941 The partial replacement of dietary methionine by cystine for purposes of growth. J. Biol. Chem., *141:* 375.

WOMACK, M., K. S. KEMMERER AND W. C. ROSE 1937 The relation of cystine and methionine to growth. J. Biol. Chem., *121:* 403.

RELATION OF CORN PRODUCTS TO THE REQUIREMENT OF THE RAT FOR DIETARY NICOTINIC ACID [1]

W. D. SALMON
Laboratory of Animal Nutrition, Agricultural Experiment Station, Alabama Polytechnic Institute, Auburn

(Received for publication August 23, 1946)

Krehl et al. ('45, '45, '45) have reported: (1) that corn products had a growth-retarding effect for rats; (2) that corn grits increased the nicotinic acid requirement of dogs; (3) that the growth-depressing effect of corn products for rats was counteracted by nicotinic acid or tryptophane or by certain proteins.

More recently Krehl et al. ('46, '46) have concluded that the relationship between nicotinic acid and tryptophane is not peculiar to corn-supplemented rations since similar effects can be demonstrated with non-corn rations which are low in both tryptophane and nicotinic acid. They report that the "deleterious action" of corn grits in a synthetic diet was prevented when fibrin, egg albumen, or soybean globulin was used in the place of casein. Moreover, the kind of carbohydrate was found to influence the extent of the "undesirable effect" of corn. These investigators suggest that the addition of corn to the diet may result in the development of unfavorable intestinal flora which may destroy nicotinic acid or that corn may contain a substance or substances which combine with nicotinic acid or in some way make it unavailable.

[1] Published with the approval of the Director of the Alabama Agricultural Experiment Station.

Any such positive harmful effects of corn would be of great nutritional importance in the South where corn bread and grits occupy such an important place in human diets. It was shown in the accompanying paper, however (Salmon, '46) that the level of casein, or tryptophane, and of fat in non-corn diets determines the need for dietary nicotinic acid by the rat. It will be shown in the following pages that this is also true in diets containing corn meal or grits and that the postulation of some kind of deleterious action peculiarly specific to corn is not supported by the data available.

METHODS AND RESULTS

The purification of the casein and the general experimental procedures were the same as in the previous investigation (Salmon, '46). The corn meal was whole white corn meal, ground on a buhr mill from locally-grown corn. The grits were the usual degerminated commercial product.

The composition of the various diets and the gains produced in rats by each diet are shown in table 1.

Corn products do not retard growth if casein level is constant

If corn had some kind of specific deleterious effect when added to a nicotinic acid-deficient diet, it would appear that the inclusion of corn in such a diet should depress the growth rate. Furthermore, one might expect that as the proportion of corn increased the depression of the growth-rate would likewise increase. The data in table 1 show, however, that the inclusion of 40% corn meal in a nicotinic acid-free basal diet, containing 9% casein, increased rather than depressed the growth rate of rats. When the corn meal was increased to 86.7%, the casein remaining at 9%, growth was substantially greater than when the diet contained only 40% corn meal. A further increase of 5% corn meal, even at the expense of the casein (corn meal 91.8%, casein 4%), resulted in a slight additional increase of growth. When casein was omitted

entirely and the diet contained only corn meal, minerals, and vitamins (no nicotinic acid included), growth was very poor but was as good as on a 9%-casein diet without corn or nicotinic acid. These data certainly fail to show any evidence of a special deleterious effect of corn meal at any level so long as the casein content of the diet is kept constant.

TABLE 1

Gains of rats receiving various levels of casein, corn products, and fat in diet[1] *for 8 weeks.*

CORN PRODUCT	CASEIN	LARD	NO NICOTINIC ACID			NICOTINIC ACID ADDED		
			Number of rats	Av. gain per rat	Gm food /gm gain	Number of rats	Av. gain per rat	Gm food /gm gain
%	%	%		gm			gm	
	9		4	22	9.45	4 [2]	63	5.09
40 Meal	9		4	55	5.96	4 [2]	200	3.48
86.7 Meal	9		4	154	3.93	4	214	3.36
91.8 Meal	4		8	168	4.27	8	170	4.48
95.8 Meal			4	21	13.50	4	22	12.73
40 Meal	9		4	41	6.41	4	201	3.60
40 Meal	9		4 [3]	188	3.86	4 [3]	212	3.36
40 Meal	9	15	4	95	3.93	4	200	3.21
40 Meal	13.3	30	4	200	2.53	4	243	2.48
40 Grits	9		4	21	14.55	4	208	3.87
40 Meal	9		4	34	7.73	4	216	3.88
40 Grits	25		4	219	3.68	4	225	3.52
40 Grits	25	15	4	247	3.14	4	248	3.02

[1] The diets contained 4.0% of Salt 5 (Salmon, '46), 0.2% of choline chloride and sucrose to make 100%; to the diets were added the following amounts of vitamin supplements in mg/kg of diet: carotene 4, α-tocopherol 50, calciferol 0.125, i-inositol 200, thiamine 2, pyridoxine 2, riboflavin 4, Ca pantothenate 10. All diets contained 0.3% of added l-cystine except the 86.7% corn meal diet which contained only 0.1%, and the 91.8% and 95.8% corn meal diets to which no cystine was added.

[2] These 2 groups received 1 mg % of nicotinic acid in the diet; the diet for all other groups receiving nicotinic acid contained 2 mg %.

[3] These 2 groups received 300 mg % of dl-tryptophane in the diet.

The addition of nicotinic acid to the 40%-corn meal-9%-casein diet increased the gain 4 to 5-fold. The addition of nicotinic acid to the 86.7%-corn meal diet also increased growth significantly; the increase was much less but total growth was slightly greater than on the 40%-corn meal diet.

When the corn meal was increased to 91.8% and the casein decreased to 4% or when the corn meal was increased to 95.8% and the casein omitted, there was no response to the addition of nicotinic acid. Obviously some factor other than nicotinic acid was limiting growth at the 91.8% and 95.8% levels of corn meal. It appears that 86.7% of corn meal furnished enough nicotinic acid to have an appreciable effect on growth.

In the absence of nicotinic acid, growth was somewhat less when corn grits was used instead of corn meal at the 40%-level; the difference may be attributable to the difference in the nicotinic acid content of the 2 products. The response to nicotinic acid was about the same on the grits diet as on the meal diet.

The addition of 15% lard to a nicotinic acid-free diet containing 40% corn meal and 9% casein doubled the rate of growth, but the addition of nicotinic acid was still necessary for maximum growth. When 30% lard was added and the casein increased to 13.3% (to maintain the same total protein: non-protein calorie ratio as in the 9%-casein, 40%-meal diet without lard), the effect on growth was comparable to the addition of nicotinic acid. Growth was only slightly increased by the addition of nicotinic acid to the 30%-lard diet. This again shows the nicotinic acid-sparing action of fat.

When the casein level was raised to 25% in a 40%-grits diet, growth was normal and there was no response to the addition of nicotinic acid. Growth was slightly increased when this diet contained 15% lard, but the effect was much less than at the lower level of casein, because the higher level of casein supplied surplus tryptophane for conversion to nicotinic acid.

Protein and fat in diet determine dietary nicotinic acid requirement of rat

The data presented in this paper confirm the findings of Krehl et al. relating to the growth-stimulating effect of nicotinic acid or tryptophane on rats receiving diets containing 40% of corn meal or grits. They do not agree with the

hypothesis that corn in some peculiarly specific manner retards growth or alters the dietary nicotinic acid and tryptophane requirement of the growing rat. Data in table 4 of the preceding paper (Salmon, '46) have shown that, in nicotinic acid-free, low-fat diets, the casein level determines the severity of the nicotinic acid deficiency; the so-called retardation of growth can be produced by substituting 6% of sucrose for 6% of casein in a 15%-casein diet. Actually, growth was slightly increased by the inclusion of 40% corn meal in the 9%-casein, nicotinic acid-free diet and tremendously increased by the inclusion of 86.7% of corn meal. If corn had any deleterious effect, it would seem that this should have increased directly with the proportion of corn in the diet.

The most significant effect of corn is its supplementary value when added to low-casein diets containing nicotinic acid. Growth was considerably greater on a 9%-casein diet that contained 40% of meal or grits than on a 12 or even an 18%-casein diet (Salmon, '46) when the diets were supplemented with nicotinic acid. It is probable that, when the supply of nicotinic acid is ample, a mixture of casein and corn proteins is superior to casein alone. The superiority is particularly evident at moderately low levels of protein.

The growth-promoting effect of 30% of lard in diets containing 40% of corn meal without added nicotinic acid was as pronounced as was shown for casein diets in the previous paper. Thus, the author's hypothesis of a nicotinic acid-tryptophane-sparing action of fat is further supported.

Obviously the amount and nature of the protein and the amount of fat in the diet determine whether the rat has a requirement for dietary nicotinic acid. When the fat content is low and the nature or amount of the protein is such that the tryptophane supply is limited, the requirement for dietary nicotinic acid is very definite. It is apparent that when corn replaces 40% of the casein in a 15% casein diet the tryptophane content of the diet is significantly lower. In the author's opinion, the problem is a deficiency problem and there is no evidence that it is aggravated by any specific deleterious effect

of corn. The effect of fat and of tryptophane upon the nicotinic acid requirement of the rat may be likened to the effect of fat on the thiamine requirement and of methionine on the choline requirement. The supposition that the rat does not require a dietary source of nicotinic acid is false unless one presupposes a liberal supply of tryptophane or fat, or both, in the diet.

SUMMARY

1. No evidence of a specific deleterious effect of corn products in nicotinic acid-deficient rations was obtained.

2. Growth of rats was very poor on a 9%-casein diet either with or without the inclusion of 40% of corn meal or grits, when nicotinic acid was not added to the diet.

3. Growth was greater with 86.7% than with 40% corn meal in a 9%-casein diet, either with or without nicotinic acid.

4. Normal growth was produced by the addition of 1 or 2 mg % of nicotinic acid to a 9%-casein diet containing 40% corn meal or grits, or 86.7% corn meal.

5. Three hundred mg % of dl-tryptophane was only slightly less effective than 1 mg % of nicotinic acid in stimulating growth on a 40%-corn meal, 9%-casein diet.

6. A 25%-casein diet containing 40% corn grits produced normal growth without the addition of nicotinic acid.

7. The inclusion of 15% lard in a 9%-casein diet containing 40% corn meal, but no added nicotinic acid, caused a significant increase in growth. Nicotinic acid was required for normal growth on this diet.

8. When 30% lard was included in the diet containing 40% corn meal and the casein increased to 13.3%, growth was normal without the addition of nicotinic acid.

9. Fat was again found to have a marked nicotinic acid-tryptophane-sparing effect, indicating that nicotinic acid functions primarily in carbohydrate metabolism.

10. The inclusion of 40% corn meal or grits in a diet, containing nicotinic acid and 9% casein, increased growth by more than 200%, thus showing a marked supplementary value of corn in low-casein diets.

11. The conditions relating to the requirement of the rat for dietary nicotinic acid are discussed.

LITERATURE CITED

KREHL, W. A., P. S. SARMA AND C. A. ELVEHJEM 1946 The effect of protein on the nicotinic acid and tryptophane requirement of the growing rat. J. Biol. Chem., *162:* 403.

KREHL, W. A., P. S. SARMA, L. J. TEPLY AND C. A. ELVEHJEM 1946 Factors affecting the dietary niacin and tryptophane requirement of the growing rat. J. Nutrition, *31:* 85.

KREHL, W. A., L. J. TEPLY AND C. A. ELVEHJEM 1945 Corn as an etiological factor in the production of a nicotinic acid deficiency in the rat. Sci., *101:* 283.

——— 1945 Effect of corn grits on nicotinic acid requirement of the dog. Proc. Soc. Exp. Biol. Med., *58:* 334.

KREHL, W. A., L. J. TEPLY, P. S. SARMA AND C. A. ELVEHJEM 1945 Growth-retarding effect of corn in nicotinic acid-low rations and its counteraction by tryptophane. Sci., *101:* 489.

SALMON, W. D. 1946 Some physiological relationships of protein, fat, choline, methionine, cystine, nicotinic acid and tryptophane. J. Nutrition, *33:* 155.

THE EFFECT OF CRUDE LECITHIN ON THE COEFFICIENT OF DIGESTIBILITY AND THE RATE OF ABSORPTION OF FAT[1, 2]

VIRGINIA AUGUR, HILDA S. ROLLMAN AND HARRY J. DEUEL, JR.
Department of Biochemistry and Nutrition, University of Southern California School of Medicine, Los Angeles

(Received for publication September 24, 1946)

It has been recognized for years that lecithin plays an important role in fat metabolism. Lecithin is an important component of the lipid fraction of blood. It is believed that when the neutral fat is transferred from the storage depot it is first converted to a water-soluble form by phosphorylation in situ and that it is carried then in the form of lecithin to the liver. Considerable amounts of lecithin are found in the liver. For these reasons it has also been suggested that lecithin may be a form into which the fat must be transformed before it can be oxidized. Verzár and Laszt ('35a, '35b) have suggested that phosphorylation of fats must occur prior to their absorption. On removal of the adrenal cortex, it was suggested that fat absorption is slowed down because of the failure of phosphorylation. The experiments of Bavetta et al. ('41) confirm the results of Verzár and Laszt in demonstrating that a decreased absorption obtains after adrenalectomy in the rat. It was also found that this effect was due to a slowing in the absorption of fatty acids inasmuch as an accumulation of the hydrolysis products of the neutral fat occurred in the

[1] Most of these data are from a thesis presented by Virginia Augur to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Master of Science.

[2] Aided by a grant from the American Lecithin Co.

intestines. However, they gave no indication as to whether the depressing action was due to a failure of phosphorylation.

Frazer ('46) has given much evidence that fats may be absorbed to a considerable extent as neutral fat provided that a fine enough emulsion is produced. If this so-called partition theory of Frazer is correct, then one might expect that any agent such as lecithin which promotes the emulsification might be of aid in speeding up the absorption of fat and in increasing the digestibility of the less readily utilized fats. If, on the other hand, the lipolytic theory is correct, then one might also postulate that the presence of lecithin would increase the speed of absorption by providing the means for producing a greater emulsification which would enable a more rapid action of the lipolytic enzymes.

The results of Adlersberg and Sobotka ('43a) have indicated that both fat and vitamin A are absorbed more rapidly in man when lecithin is added to the diet. The rise in total lipids of the serum on a protein-free diet without lecithin was only 32% after 4 hours, compared with 71% in subjects on a similar diet who received lecithin along with the food. When 120,000 or 180,000 International Units (I.U.) of vitamin A were given as a component of percomorph oil, there was a rise in vitamin A content of blood plasma of only 41% when given without lecithin or an average of 212% increase if given along with lecithin. In addition, lecithin has an effect on the level of blood cholesterol. It was demonstrated by Adlersberg and Sobotka ('43b) that the prolonged administration of lecithin brought about a slight decrease in serum cholesterol, although it returned to high levels after the administration of lecithin was stopped. Similar results are recorded by Steiner and Domanski ('44), although the latter investigators found that the high levels of blood cholesterol returned after 4 or 5 weeks in spite of continued lecithin feeding. Urbach ('46), however, was able to maintain persistently low levels of blood cholesterol when a diet low in carbohydrate and fat was given along with the lecithin. The results of Adlersberg were also confirmed by Gross and Kesten ('43). Slanetz and Scharf

('43, '44) have reported that some unknown factor is necessary for the utilization of vitamin A in the rat which is present in crude lecithin and which is not vitamin E.

The total availability of the fat (i.e., the coefficient of digestibility) as well as the rate of absorption are both important in evaluating the nutritive value of fat. Although the coefficient of digestibility of poorly utilized fats and their rate of absorption are roughly parallel, differences in rate of absorption may occur in fats which are practically completely digestible. Whereas the coefficient of digestibility may be obtained by calculation from the fat ingested and that excreted in the feces, the rate of absorption can only be determined by short term tests where the actual speed of removal of the lipid from the small intestine is ascertained.

Because most of the work which has been done on the effect of lecithin on the absorption of fat has been indirect rather than direct, the present experiments were undertaken to determine whether an increased rate of absorption occurs with fats when mixed with lecithin and also whether any improved digestibility of fats obtains when additional lecithin is added.

EXPERIMENTAL

The effect of lecithin on digestibility was demonstrated by feeding rats on diets containing approximately 15% fats of high melting point with or without added lecithin. The animals were allowed a 2-day period for orientation and the feces were then collected for the following 8 days. Food consumption was recorded during this period and the diets were allowed ad libitum. At the conclusion of the digestibility experiment, the animals were fasted for 2 days after which the effect of lecithin on the rate of absorption was determined for 3- and 6-hour periods. The same fats fed on the digestibility experiments were used in the absorption tests. The diets employed are recorded in table 1.

The combined feces of the 8-day period were dried under a vacuum to constant weight, ground with a mortar and pestle and the neutral fat extracted on a Soxhlet extractor with

diethyl ether. The residue was again dried, mixed with a minimum amount of 50% sulfuric acid and the acid paste reextracted with ether. The neutral fat and fatty acid obtained were dried under a vacuum at 60°C. to constant weight. It was demonstrated that no acid could be extracted by diethyl ether from sulfuric acid solution and it was also found that the material extracted from dried feces after acidification was acid in reaction and possessed the properties of a fatty acid.

TABLE 1

Composition of diets used in digestibility studies.

COMPONENTS OF DIET	DIET 1	DIET 2	DIET 3
	%	%	%
Commercial casein	18	18	18
Glucose	56	53	71
Hydrogenated cottonseed oil	15	15	0
Salt mixture [1]	7	7	7
Yeast [2]	1	1	1
Liver extract [3]	3	3	3
Crude Soya Lecithin [4]	0	3	0

[1] Osborne-Mendel ('19).

[2] Anheuser-Busch, Strain G.

[3] Wilson and Co., 1 : 20 concentrate.

[4] Prepared by The American Lecithin Co.

The calculations for digestibility were made in the usual way, correction being made for the metabolic fat. The correction for metabolic fat was determined in experiments where the animals were fed on Diet 3 (table 1) in which the fat has been replaced by glucose. The 10 rats used in this determination weighed on an average, 209 gm, consumed a total of 94.5 gm of food, and excreted stools which had an average dried weight of 5.35 gm. The feces contained a total of 181 mg of fat as neutral fat, 87.5 mg as soap which makes a total of 268 mg or 50.5 mg per gm of dried stool. The latter figure was used in the subsequent calculations. The coefficient of digestibility was expressed as the per cent of the ingested fat which had been utilized. The amount of digested fat was

determined by subtracting the corrected excretion value from the original weight of ingested fat.

The absorption tests were carried out by the usual procedure as described in the paper of Deuel, Hallman and Leonard ('40). The fat was fed at a level of 300 mg per 100 cm^2 of body surface based on fasted weight. Surface area was estimated by the formula of Lee ('29). In some preliminary experiments where the effect of lecithin on preventing diarrhea was determined after giving large doses of cottonseed oil, the fats were fed at approximately 1.5 or 2 times the usual level. The experiments were carried out on a commercial sample of cottonseed oil [3] and on 3 samples of cottonseed oil hydrogenated to melting points of 46°, 54°, and 65°C.[4] The crude lecithin was added in the absorption and diarrhea tests in proportions of one-sixth or one-fifth, respectively. According to the manufacturer, the crude lecithin contains 30 to 40% of soybean oil and about 60 to 70% of mixed phosphatides of lecithin, cephalin and lipositol. Adult female rats were used throughout the experiment except in one series of tests on susceptibility to diarrhea and were from the University of Southern California colony.

RESULTS

In a series of preliminary experiments, tests were made to determine whether a greater amount of fat could be digested without the onset of diarrhea if lecithin was added to the diet. A summary of these experiments is recorded in table 2.

When either cottonseed oil or hydrogenated cottonseed oil was fed at several levels, the number of rats which developed diarrhea was invariably higher in the group where no lecithin was added. In a grand total of 50 experiments where fat was fed without lecithin, a total number of 20 cases of diarrhea were noted which is an incidence of 40%. Only 4 rats of the 40 which received lecithin with the fat developed a diarrhea,

[3] Wesson Oil.

[4] These fats were kindly prepared for us by Dr. K. Mattil of Swift and Co.

TABLE 2

The incidence of diarrhea in rats fed several fats with or without added lecithin after a previous 48-hour fast period.

FAT FED	LENGTH OF PERIOD	FAT ALONE				FAT PLUS LECITHIN			
		Sex	Dose	No. of tests	No. with diarrhea	Sex	Dose	No. of tests	No. with diarrhea
	hours		*mg/ 100 cm²*				*mg/ 100 cm²*		
Cottonseed oil	3	F	328	3	1				
	3	F	410	4	3	F	410	7	0
	2	F	410	23	5	F	410	16	1
	2	M	615	10	4	M	615	10	2
Hydrogenated cottonseed oil	6	F	337	10	7	F	374	7	1

TABLE 3

Summary table of digestibility of 3 samples of cottonseed oil hydrogenated to different degrees when fed in a diet without lecithin (Diet 1) or with lecithin (Diet 2).

DATA SUMMARIZED	FAT 1 (46°C.)		FAT 2 (54°C.)		FAT 3 (65°C.)	
	Diet 1	Diet 2	Diet 1	Diet 2	Diet 1	Diet 2
Number of rats	10	10	9	10	10	10
Average weight of rats, gm	191	157	173	181	241	243
Average gain, gm	6.5	12.3	10.3	8.0	0.9	— 1.4
Fat in diet, %	16.6	20.2	15.7	20.7	17.6	18.4
Average food eaten, gm	88	86	82	83	90	91
Average fat ingested, gm	14.9	17.5	12.3	17.2	15.7	16.6
Average weight of dried stools, gm	7.8	6.7	9.1	8.5	17.7	15.2
Average fat excreted						
As neutral fat and fatty acid, gm	0.73	0.62	0.62	0.79	6.31	4.83
As soaps, gm	2.09	1.86	3.80	2.63	6.93	5.33
Total, corrected [1]	2.39	2.09	3.92	2.95	12.25	9.32
Coefficient of digestibility [2]	83.8 ± 1.4	87.9 ± 0.7	68.7 ± 2.7	82.8 ± 1.4	24.0 ± 2.6	44.2 ± 2.0
M.D. : S.E.M.D.[3]		2.66		4.71		6.05

[1] Corrected for metabolic fat by multiplying weight of dried stools by 50.5 mg.

[2] Including the standard error of the mean calculated by the formula $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

[3] Mean Difference : Standard Error of Mean Difference of results on Diet 1 compared with those on Diet 2. When this value exceeds 3, the results are considered significant.

which equals only 10%. In the cottonseed oil tests, the experiments with and without lecithin were carried out simultaneously. In the case of the hydrogenated cottonseed oil, the experiments on lecithin were carried out several days later on the 7 rats which had developed diarrhea when cottonseed oil alone was fed. The data on the digestibility of various samples of cottonseed oil are summarized in table 3.

The addition of lecithin considerably increased the digestibility of the cottonseed oil, particularly in the case of the

TABLE 4

Summary table of absorption rates of cottonseed oil, of 2 samples of hydrogenated cottonseed oil when fed (A) without or (B) with lecithin.

FAT (A) WITHOUT OR (B) WITH LECITHIN	NO. OF TESTS	DURATION	AVERAGE RAT WEIGHT	AVERAGE FAT IN MG				M.D. : S.E.M.D.[3]
				Fed	Recovered [1]	Absorbed		
						Total	Per 100 cm[2] per hr [2]	
				Cottonseed oil				
		hrs.	gm					
A	11	2	135	911	693	238	47.8 ± 2.3	
B	14	2	132	908	635	292	61.8 ± 3.7	3.21
A	6	3	112	705	488	237	38.5	
B	8	3	121	835	460	395	59.9	
			Hydrogenated cottonseed oil (m.p. 46°C.)					
A	10	3	200	864	601	293	26.5 ± 1.8	
B	10	3	204	925	476	479	48.9 ± 3.0	3.56
A	11	6	169	775	403	402	24.7 ± 2.0	
B	9	3	207	946	323	643	36.7 ± 2.2	4.12
			Hydrogenated cottonseed oil (m.p. 54°C.)					
A	9	3	197	822	674	178	18.0 ± 1.8	
B	8	3	189	837	587	280	30.1 ± 2.8	3.58
A	9	6	197	836	709	157	8.5 ± 0.9	
B	9	6	198	870	519	381	21.8 ± 1.5	7.65

[1] Does not include the correction for lipid content of fasting rats. The value used for the cottonseed oil tests was 20 mg, and for the hydrogenated cottonseed oil tests, 30 mg.

[2] Including the standard error of the mean calculated by the formula $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

[3] Mean Difference : Standard Error of Mean Difference of results on "A" compared with "B." When this value exceeds 3, the results are considered significant.

highest melting sample. The low digestibility of the fat melting at 63°C. is not to be ascribed to a failure of lipolysis but to the inability to absorb the stearate so formed. We have no evidence from our tests on how large a proportion of the material which was ether-soluble before acidification was neutral fat and how much represented the free fatty acid. The total stool weight was slightly lower in all cases with the lecithin-fed rats.

The results of the absorption tests with cottonseed and hydrogenated cottonseed oils are recorded in table 4.

DISCUSSION

There would seem to be little doubt that the addition of a crude lecithin preparation to fat lessens the susceptibility to diarrhea in rats. This effect might be the resultant of a depressing action of some components of the crude lecithin on gastric or intestinal motility or secondly because it causes a more rapid rate of absorption whereby the quantity of fat present in the gut is sufficiently reduced to lessen the chance of an ensuing diarrhea.

The present experiments indicate that the above effect is probably to be traced to an acceleration in the absorption rate. In the case of cottonseed oil the rate of increase in absorption is 29% at 2 hours and 56% at 3 hours. With the hydrogenated fat melting at 46°C., the results are equally striking, being 85 and 49% at 3 and 6 hours, respectively. The increased absorption rates for the higher melting fat (54°C.) containing lecithin are 67 and 156%, respectively, for the corresponding periods.

There are several possible explanations as to why crude lecithin may increase the absorption rate. In the first place, it might be suggested that lecithin is preferentially absorbed from the fat-lecithin mixture at a faster rate than the balance of the fat. It is difficult to see how this could occur from the lecithin-fat mixtures which are entirely homogenous. Moreover, if one assumes a 100% absorption of the lecithin in all 4 series of tests with the hydrogenated cottonseed oil samples,

there still would be an additional absorption of the neutral fat in the lecithin groups over those experiments where lecithin was not administered. In the cottonseed oil tests, the increase is approximately equivalent to the lecithin fed.

Secondly, it may be argued that the lecithin can not be recovered from the gut by the flushing procedure with diethyl ether. In tests where the recovery of known amounts from the gastro-intestinal tract was tested by removing the gut immediately after the fat had been discharged into it, it was found that the average recovery after cottonseed oil was 95.4 and after cottonseed oil-lecithin mixture, 92.9%.

Thirdly, it is possible that lecithin promotes absorption by increasing the speed and degree of emulsification. Such an effect could be explained either by an increased rate of absorption of unhydrolyzed triglyceride due to increased emulsification (according to the Partition Hypothesis of Frazer), or that an increased speed of the action of the lipolytic enzymes is possible because of the greater surface for their action.

The action of lecithin in increasing digestibility must be related to its speeding up of absorption. With the large amount of fat available at one time in the intestine, it is impossible for it to be removed from the gut at the very slow rate that absorption normally obtains before it has passed beyond the absorptive portion of the gastro-intestinal tract. Anything which will increase the rate of absorption during the interval that it is in the area where it can be absorbed will obviously improve the digestibility.

SUMMARY

The addition of lecithin to cottonseed oil or to a hydrogenated cottonseed oil markedly lowers the susceptibility to diarrhea caused by a large dose of these fats to rats.

Fats containing one-sixth or one-fifth crude lecithin are absorbed more rapidly than a similar fat without any added phosphatide.

It was found that hydrogenated cottonseed oil melting at 63°C. had a digestibility of 24 in the rat; that melting at

54°C. was digested to the extent of 69% while that with a melting point of 46°C. had a digestibility coefficient of 84. These were increased by the addition of lecithin to 44, 83 and 88%, respectively.

A considerably larger portion of the lipid in all cases was excreted as soaps than as neutral fat plus fatty acids.

LITERATURE CITED

ADLERSBERG, D., AND H. SOBOTKA 1943a Influence of lecithin feeding on a fat and vitamin A absorption in man. J. Nutrition, *25:* 255.

———— 1943b Effect of prolonged lecithin feeding on hypercholesterolemia. J. Mount Sinai Hosp., *9:* 955.

BAVETTA, L., L. HALLMAN, H. J. DEUEL, JR. AND P. O. GREELEY 1941 The effect of adrenalectomy on fat absorption. Am. J. Physiol., *134:* 619.

DEUEL, H. J., JR., L. HALLMAN AND A. LEONARD 1940 The comparative rate of absorption of some natural fats. J. Nutrition, *20:* 215.

FRAZER, A. C. 1946 The absorption of triglyceride fat from the intestine. Physiol. Rev., *26:* 103.

GROSS, P., AND B. KESTEN 1943 Treatment of psoriasis with lipotropic substances derived from foodstuffs. Arch. of Dermat. and Syph., *47:* 159.

LEE, M. O. 1929 Determination of surface area of the white rat with its application to the expression of metabolic results. Am. J. Physiol., *89:* 24.

OSBORNE, T. B., AND L. B. MENDEL 1919 Nutritive value of wheat kernel. J. Biol. Chem., *37:* 572.

SCHARF, A., AND C. SLANETZ 1944 Effect of soybean phosphatides on utilization of the fat-soluble vitamins. Proc. Soc. Exp. Biol. Med., *57:* 159.

SLANETZ, C., AND A. SCHARF 1943 The effect of phosphatides on utilization of vitamin A and carotene. Proc. Soc. Exp. Biol. Med., *53:* 17.

STEINER, A., AND B. DOMANSKI 1944 Effect of feeding of "soya lecithin" on serum cholesterol level of man. Proc. Soc. Exp. Biol. Med., *55:* 236.

URBACH, E. 1946 Skin Diseases, Metabolism and Nutrition. Grune and Stratton, New York, p. 542.

VERZÁR, F., AND L. LASZT 1935a Die Hemmung der Fettresorption nach Exstirpation der Nebennieren. Biochem. Ztschr., *276:* 11.

———— 1935b Nebennierenrinde und Fettresorption. Biochem. Ztschr., *278:* 396.

A COMPARISON OF THE COEFFICIENT OF DIGESTIBILITY AND THE RATE OF ABSORPTION OF SEVERAL NATURAL AND ARTIFICIAL FATS AS INFLUENCED BY MELTING POINT [1,2]

MARY E. CROCKETT AND HARRY J. DEUEL, JR.
Department of Biochemistry and Nutrition, University of Southern California, School of Medicine, Los Angeles

(Received for publication September 24, 1946)

Fats and oils offer an excellent source of energy. Nearly all of the natural fats have been found to be from 93 to 98% digestible (Langworthy, '23). The only exceptions found in the case of man are those with high melting points and those which contain irritants such as castor and croton oil.

It has been suggested that an inverse relationship exists between the coefficient of digestibility and the melting point of the fat (Holmes and Deuel, '21). The critical temperature above which there is a marked decrease in digestibility in man appears to be about 50°C. Thus, it was found that mutton fat (Langworthy and Holmes, '15) and deer fat (Deuel and Holmes, '22) are digested to an extent of only 88 and 81.7%, respectively. Hydrogenated fats (Holmes and Deuel, '21) and blended hydrogenated fats (Deuel and Holmes, '22) also have a lower digestibility in man when the melting point exceeds 50°C. More recently it has been stated by Mattil

[1] The data are from a thesis presented by Mary E. Crockett to the Graduate School of The University of Southern California in partial fulfillmen of the requirements for the degree of Master of Science.

[2] Aided by a grant from Swift and Co.

and Higgins ('45) that the stearate content rather than melting point is the prime consideration in determining the completeness of digestibility. Inasmuch as the higher melting points of fat are usually associated with an increased proportion of tristearin, it is difficult to study these variables separately.

The nutritive value of fat can also be established by determining its rate of absorption from the gastro-intestinal tract. It is possible that a fat which is almost completely digested over a given length of time, might nevertheless have a slow rate of absorption through the intestinal wall. There are very little data at present on the rate of absorption of fats. Steenbock and others ('36) found a significant difference between the absorption rate of butter fat and coconut oil, whereas Deuel et al. ('40) found no consistent differences. This was probably due to the units employed for expressing rate of absorption. Deuel et al. ('40) found that uniform results were obtained when comparison of absorption rate was made on the basis of body surface area.

The present experiments were designed to compare the digestibility of several of the commercially hydrogenated products in the rat with other natural fats and to determine whether a satisfactory digestibility occurs with a blended hydrogenated fat, namely bland lard.[3] The tests were made on rats since the digestibility of fats in this animal is particularly effected by a high melting point. The tests were also designed to determine whether the rate of absorption is altered before the digestibility. A further discussion of the importance of terms "digestibility" and "absorption" as used in this laboratory is given in the earlier paper (Augur et al., '47).

EXPERIMENTAL AND RESULTS

Digestibility and absorption experiments were carried out on a commercial margarine made from hydrogenated vegetable

[3] Bland lard is a blended hydrogenated fat composed of a mixture of lard hydrogenated to a high melting point and deodorized lard containing an antioxidant.

TABLE 1

Summary table of digestibility of hydrogenated fats when fed to female rats in a diet at a level of 15%.

DATA SUMMARIZED	MARGARINE	CRISCO	BLAND LARD	PRIME STEAM LARD	HYDROGENATED LARD	HYDROGENATED LARD
Melting point, °C.	34	43	48	37	55	61
Number of rats	20	20	18	16	18	20
Average weight of rats, gm	214	207	208	229	244	223
Average gain, gm	—1	+5	+1	+2	—4	—6
Average food eaten, gm	72	82	85	81	90	88
Average fat ingested, gm	10.7	12.3	12.8	12.2	13.5	13.2
Average weight of stools, gm	4.40	5.50	5.86	5.36	10.0	15.0
Average fat excreted						
As neutral fat and fatty acids, gm	0.24	0.23	0.34	0.21	0.58	2.21
As soaps, gm	0.30	0.38	0.68	0.48	4.90	8.89
Average total fat excreted (corrected), gm [1]	0.31	0.33	0.72	0.39	4.94	10.34
Coefficient of digestibility [2]	97.0 ±0.4	97.3 ±0.3	94.3 ±1.8	96.6 ±1.4	63.2 ±1.2	21.0 ±2.6

[1] Corrected for metabolic fat by multiplying weight of dried stools by 50.5 mg.
[2] Including the standard error of the mean calculated by the formula $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

oils, Crisco, bland lard,[4] prime steam lard,[4] and lards hydrogenated to 55° and 61°C.[4] The diets and the methods of analyses were entirely similar to those employed in the previous study (Augur, Rollman and Deuel, '47). The tests were made on adult female rats from the University of Southern California colony.

[4] These fats were kindly furnished us by Swift and Co. The samples of hydrogenated lard were prepared for us by Dr. K. Mattil of that organization. The authors wish to thank Mr. J. H. Luckmann of The Best Foods, Inc., for checking the melting points.

TABLE 2

Summary table of absorption rate of 5 samples of fat.

DATA SUMMARIZED	MARGARINE		CRISCO		BLAND LARD		PRIME STEAM LARD		HYDROGENATED LARD — 55°C.	
	3-hour tests	6-hour tests	3-hour tests	6-hour tests	3-hour tests	6-hour tests	3-hour tests	6-hour tests	3-hour tests	6-hour tests
No. of rats	10	10	10	10	10	10	10	10	12	10
Average fasting weight, gm	198	202	198	200	177	185	227	200	209	216
Average fat fed, mg	875	889	875	874	809	835	950	883	906	898
Average fat recovered, mg [1]	519	240	516	217	494	254	590	166	688	482
Average fat absorbed, mg, total	356	649	358	657	315	581	360	717	218	416
Per 100 cm^2/hr. [2]	38.7 ±1.5	34.7 ±1.0	37.1 ±2.0	34.3 ±0.6	34.5 ±1.7	31.4 ±1.6	35.0 ±1.9	38.3 ±2.02	20.7 ±3.5	21.6 ±3.5

[1] Correction for lipid content of intestine of 2-day fasted rats. 36.3 mg was subtracted from the amount recovered for each test.

[2] Including the standard error of the mean calculated by the formula $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

Digestibility studies

Sixteen to 20 digestibility experiments were made on each fat. The average results are summarized in table 1.

There are no significant differences in the coefficient of digestibility between the fats with melting points less than 55°C. The range is from 94.3% for bland lard to 97.3% for Crisco. The hydrogenated lards with a melting point of 55°C. and above show a marked decrease in digestibility. The weight of the stools increased with the increase in melting point. The lack of digestibility of the hydrogenated lards is not due to a failure of lipolysis but to the inability to absorb the palmitate and stearate formed from this action.

Absorption tests

The average results of the absorption studies are given in table 2. The rats were fed the same fats as used in the digestibility studies. No tests were possible on the hydrogenated lard melting at 61°C. since the temperature of the liquid phase was high enough to cause injury. No accurate method of feeding it as an emulsion was found whereby a quantity comparable to that of the other fats could be fed.

DISCUSSION

Margarine, Crisco, prime steam lard and bland lard have all been shown to be practically completely digestible in the rat and to compare favorably with natural fats and oils in which the melting point is below 50°C. The value for margarine is identical with that recently reported in human subjects (Deuel, '46) and that for prime steam lard is almost identical with that found by Langworthy and Holmes ('15) in man. As would be expected, the hydrogenated lard samples melting at 55°C. and 61°C. were much less digestible than other fats. In experiments on man, practically no decrease in digestibility obtained with hydrogenated vegetable fats melting at 50°C. (Holmes and Deuel, '21) but with rats only a 63% utilization occurred. In fact, with hydrogenated fats

melting as high as 52.4°C. (Deuel and Holmes, '22), the lowest digestibility found with man was 79.

The discrepancy between the results on man and the rat may be a species difference. Herbivora digest high melting fats much less satisfactorily than omnivora or carnivora (McCay and Paul, '38) but are able to utilize castor oil as well as other fats (Paul and McCay, '42). On the other hand, the variation may be due to the technic employed. In the tests on human subjects, the feces were extracted with diethyl ether without acidification whereby only the neutral fat and free fatty acids would be recovered. Any fatty acid in the form of soap would be lost. In the present tests after extraction of the neutral fat and fatty acids, the residue was acidified and reextracted with diethyl ether whereby the fatty acids formed from the soaps would then be extracted. While the quantity present as soap is very small in the stools after such completely digestible fats as margarine, Crisco, and the two lard samples were fed, it amounted to 4 to 6 times the quantity of undigested neutral fat plus free fatty acid when the hydrogenated lards were fed. With the exception of Hoagland and Snider ('43), the previous investigators have not generally taken the soaps into account. It is possible that if the tests on humans are made using a similar technic, a considerable amount of soap might be found with the high melting fats and a greatly decreased digestibility would be demonstrated.

The failure in absorption of the high melting fats cannot be due to the inadequacy of lipolysis. The fatty acids set free far exceed that required to produce a satisfactory emulsion (when combined with monoglyceride and bile salts) according to the Particulate Hypothesis of Frazer ('46). It is possible that emulsification is incomplete with the high melting fats or that the fat particles may be too large to be absorbed through the intestinal mucosa.. The improved utilization of hydrogenated cottonseed fat when lecithin is mixed with it is demonstrated by Augur et al. ('47) may be the result of improved emulsification with increased absorption of neutral fat.

The high digestibility of the bland lard which contains a portion of the completely hydrogenated lard indicates that the digestibility of high melting fat is considerably increased when fed as a component of a blended fat having a melting point in the physiological range. It is similar to the results obtained on human subjects with blended fats (Deuel and Holmes, '22) although no account was taken in these earlier tests of the possible excretion of fat as soaps.

There is a decrease in rate of absorption of the hydrogenated lard melting at 55°C. compared with the lower melting fats which is proportional to the decrease in digestibility. Thus the decrease in absorption rate for the hydrogenated lard (m.p. 55°C.) compared with prime steam lard was 41% while the digestibility was lowered by 35%.

There was no diarrhea resulting from the feeding of rats diets containing 15% of the different fats over a 10-day period. Also no diarrhea occurred in the absorption tests. In the digestibility tests, the rats appeared to be normal at the end of the experiment and no upset in nutritional condition was noted.

SUMMARY

1. The digestibility of various fats has been studied. The digestibility coefficients obtained were margarine (m.p. 34°C.), 97.0; Crisco (m.p. 43°C.) 97.3; prime steam lard (m.p. 37°C.), 96.6; bland lard (m.p. 48°C.), 94.3; hydrogenated lard (m.p. 55°C.), 63.2; and hydrogenated lard (m.p. 61°C.), 21.0.

2. There was no evidence that any fat produced any abnormal physiological effect, such as diarrhea.

3. The rates of absorption for 3 hours, expressed in mg per 100 cm^2 per hour, were: margarine, 37.7; Crisco, 37.1; prime steam lard, 35.0; bland lard, 34.5; hydrogenated lard (m.p. 55°C.), 21.6. The values obtained for 6 hours were: margarine, 34.7; Crisco, 34.3; prime steam lard, 38.3; bland lard, 31.4; hydrogenated lard (m.p. 55°C.), 21.6.

4. A large proportion of the undigested fat in the tests with the hydrogenated lards was excreted in the form of soaps.

LITERATURE CITED

AUGUR, V., H. S. ROLLMAN AND H. J. DEUEL, JR. 1947 The effect of crude lecithin on the coefficient of digestibility and the rate of absorption of fat. J. Nutrition, *33:* 177.

DEUEL, H. J., JR. 1946 Studies on the comparative nutritive value of fats. IX. The digestibility of margarine fat in human subjects. J. Nutrition, *32:* 69.

DEUEL, H. J., JR., AND A. D. HOLMES 1922 The digestibility of cod liver, Java almond, tea seed, and watermelon seed oils, deer fat and some blended hydrogenated fats. U.S. Dept. Agric. Bull. 1033.

DEUEL, H. J. JR., L. HALLMAN AND A. LEONARD 1940 The comparative rate of absorption of some natural fats. J. Nutrition, *20:* 215.

FRAZER, A. C. 1946 The absorption of triglyceride fat from the intestine. Physiol. Rev., *26:* 103.

HOAGLAND, R., AND G. G. SNIDER 1943 Digestibility of some animal and vegetable fats. J. Nutrition, *25:* 295.

HOLMES, A. D., AND H. J. DEUEL, JR. 1921 Digestibility of some hydrogenated oils. Am. J. Physiol., *54:* 479.

LANGWORTHY, C. F. 1923 The digestibility of fats. Ind. and Eng. Chem., *15:* 276.

LANGWORTHY, C. F., AND A. D. HOLMES 1915 Digestibility of some animal fats. U.S. Dept. Agric. Bull. 310.

McCAY, C. M., AND H. PAUL 1938 The effect of melting point of fat upon its utilization by guinea pigs. J. Nutrition, *15:* 377.

MATTIL, K. S., AND J. W. HIGGINS 1945 The relationship of glyceride structure to fat digestibility. I. Synthetic glycerides of stearic and oleic acids. J. Nutrition, *29:* 255.

PAUL, H., AND C. M. McCAY 1942 Utilization of fats by Herbivora. Arch. Biochem., *1:* 247.

STEENBOCK, H., M. H. IRWIN AND J. WEBER 1936 The comparative rate of absorption of different fats. J. Nutrition, *12:* 103.

THE USE OF ALBINO MICE TO DETERMINE THE UTILIZATION OF THE CALCIUM OF DEHYDRATED CARROTS AND CABBAGE

ADELAIDA M. BENDAÑA-BROWN AND BARKER H. BROWN
Department of Biological Chemistry, School of Medicine,
University of Michigan, Ann Arbor

(Received for publication July 19, 1946)

The literature on the availability of calcium in vegetables has been reviewed briefly by Fincke and Sherman ('35) and Fincke ('41). Most of this work has been carried out either by balance studies on human beings or by deposition experiments using weanling albino rats with the comparison of the utilization of a given foodstuff to that of milk.

The availability of dietary calcium, in foods other than dairy products, assumes a far greater importance in those regions with a negligible dairy industry such as the Far East where the authors have been for several years. One of us, Bendaña-Brown, was studying the availability of calcium in various Philippine foodstuffs in Manila from 1938 until the capture of the city in January, 1942. Preliminary results by Bendaña-Brown and Geronimo [1] ('41) showed that the calcium of pechay, a Philippine vegetable similar to Chinese or celery cabbage with a high calcium content (about 1% when dried), had an availability of 75 to 90% of that of dried skim milk, and that soy bean curd cheese (tokua) had an availability of about 80 to 90% of that of dried skim milk when tested by the rat deposition method of Fincke and Sherman ('35) at a dietary calcium level of 0.26%. Kung, Yeh and Adolph ('38), feeding rats at a dietary level of 0.10% cal-

[1] Killed in the reoccupation of Manila in February, 1945.

cium, found 95 to 99% utilization of the calcium of celery cabbage while Kao, Conner and Sherman ('38) found a value of about 80% when half of the calcium of the milk diet was replaced by the calcium of Chinese cabbage at a dietary calcium level of 0.26 to 0.28%. Adolph and Chen ('32) working with 3 Chinese adults in balance experiments, found that soy bean curd was approximately equal to milk as a source of calcium. Potgieter ('40a, '40b), in Hawaii, found that a diet in which 97% of the calcium was furnished by taro was well utilized by both women and growing rats. Potgieter ('40a), using rats in paired feeding experiments with $Ca(H_2PO_4)_2 \cdot H_2O$ as a control and feeding at levels of dietary calcium below 0.10% with taro furnishing 97% of the dietary calcium, found that 82% of the taro calcium and 88% of the inorganic salt calcium was utilized. The same author ('40b), testing 1 Caucasian woman and 1 Japanese woman, found that the latter would maintain calcium balance on a lower calcium level than the former. However, Steggerda and Mitchell ('46) have shown a considerable normal variation (23%) in the calcium metabolism and requirements of normal Caucasian adults. Lunde and Lie ('40) and Basu, De and Basak ('42) have found that the calcium of the bones of small fish possesses about the same availability as that of milk. MacDonald and Bose ('42), have found that egg shells are beneficial to rats fed on a diet similar to that eaten by the poor Indian population. In the Santo Tomas Internment Camp in Manila, during the Japanese occupation, a number of us supplemented our meager rice and corn diet with pulverized egg shells partially dissolved in vinegar. Basu, Basak and De ('42) found that when 6 betel nut leaves were chewed with lime (a portion of a betel nut plus the rolled leaves and a portion of slaked lime and often some tobacco are all chewed together producing a blood red sputum which stains the lips and teeth), calcium equivalent to 10 oz. of milk was available and that the calcium so obtained was well retained and utilized. In the Philippines this habit is widely practiced and considered to be of particular value during

pregnancy. Kelly ('43) has found for rats utilization values of 80% to 86% for milk, 37% for beet greens, 70% for Swede turnips, 80% for Savoy cabbage, and 76% for parsnips. Eighty-six per cent of the calcium intake in these experiments was derived from the test food which was fed fresh, raw, and uncooked at a suboptimal level. Breiter, Mills, Rutherford, Armstrong and Outhouse ('42), in balance experiments with 4 men and 3 women, found utilization values of from 0.0 to 35.5% (average 13.4%) for the calcium from carrots. These same subjects had previously been used by Breiter, Mills, Dwight, McKay, Armstrong and Outhouse ('41) for similar experiments with milk where the values obtained were appreciably higher in 5 of the 7 cases.

The authors felt that a more rapid method of biological determination of the relative utilization of calcium of various sources would be of value. In an effort to bring this about, the following work with albino mice was undertaken. We wish to express our sincere appreciation to Prof. H. B. Lewis and his staff for facilities and advice during our work which was terminated by the necessity of an early return to Manila.

EXPERIMENTAL

The method used is based on that introduced by Fincke and Sherman ('35) for rats. Albino mice were weaned at 18 to 21 days of age and placed on the various experimental diets, the composition of which is given in table 1. The diets for a given experiment were all prepared at the beginning of the experimental period and a sample of each was ashed and analyzed for calcium.

Litter mates of the same sex were used in the comparison of the various diets. The animals of each litter group were placed on the diets as indicated in the tables and one or more of each litter were killed at the beginning of the experiment for calcium analysis as a base line control of the calcium content of that litter at the beginning of the feeding period. The median weight mouse in each litter was used for the base line control and the one nearest to it in weight served as the

experimental control on the basic 20% dried skim milk diet (diet M-2a). The mice were kept in individual metabolism cages with raised screen bottoms. Records were kept of the food consumed. The experiments were continued until the animals doubled or tripled their initial weights which was in general at about 40 days of age or after a feeding period of 18 to 21 days. They were then killed with chloroform

TABLE 1

Percentage composition and calcium content of the experimental diets.[1]

DIET NO.	GROUND POLISHED RICE	DRIED SKIM MILK	CORN STARCH	DEHYDRATED CARROTS	DEHYDRATED CABBAGE	CALCIUM CONTENT
						%
M-2	65	20	1			0.279
M-3 [2]	65	15	5.5			0.281
M-4 [3]	65	10	10			0.280
M-5	60	25	1			0.343
M-6 [2]	65	20	0.5			0.345
M-2a	65	20				0.280
Cab-1	47	10			28	0.276
Cab-2	52	13			20	0.281
Cab-3	57	16			12	0.282
C-3	34	13		38		0.278
M-3b	70	15				0.222
M-4b	75	10				0.172
M-5a	60	25				0.343

[1] In addition to the constituents listed, all diets contained 1.5% sodium chloride (C.P.), 0.5% of dried beef liver (prepared in this laboratory), 2% of dried yeast powder (Mead Johnson), 2% of cod liver oil and 9% of corn oil (8% in diets M-2 to M-6, inclusive).

[2] 0.5% of calcium lactate added.

[3] 1.0% of calcium lactate added.

and the bodies, minus the digestive tract, were ashed in platinum dishes and analyzed for calcium by the A.O.A.C. volumetric method ('40) with minor modifications.

In order to minimize the labor involved and to obtain the most consistent results, it was found advisable to provide a 5-oz. wide mouth bottle for each experimental animal and to weigh into this at the beginning of the experiment the food

for the entire period, usually about 60 gm. These bottles were kept in the refrigerator and food was transferred to the food cups daily and mixed with water to a paste which almost entirely eliminated scattering. With a little practice, it was possible to adjust the amounts of food dispensed daily so that little or none remained and the over-all labor saved was considerable. Since the only weighings of food necessary were those at the beginning and the end of the experiment, it was possible to feed 25 to 30 experimental animals in 30 to 40 minutes daily.

The cabbage and carrots used were dehydrated vegetables obtained for us by Prof. H. B. Lewis through the courtesy of Major George Berryman of the Chicago Medical Nutrition Laboratory of the Armed Service Forces and Colonel John B. Youmans. Each vegetable and the mixed diets were analyzed for calcium, all materials being finely ground and mixed thoroughly before sampling for analysis.

DISCUSSION AND RESULTS

In planning our diets (table 1), it seemed advisable to use diets which could be easily reproduced either in this country or in the Philippines for future work. This made it desirable to replace whole wheat with polished rice. Rice had the further advantage of being very low in calcium (0.009%) so that the amount of rice could be varied without materially affecting the calcium content of the diet. The previous work of Fincke and Sherman ('35) shows that, when rather large amounts of dried vegetables are used, the protein content of the experimental diets often exceeds that of the skim milk control diet. The replacement of the corn starch of the Fincke and Sherman diet with rice of 8% protein content tends to give control and experimental diets of a more nearly equal protein content.

As there were no available data on mice, an attempt was made in the first experiments to determine whether or not the procedure of feeding a 20% dried skim milk diet as a control and replacing one-half of the milk with an equivalent

amount of calcium from the source to be tested, as the Sherman group have done with rats, was applicable to this other species. It was felt that a study of the utilization of calcium at various levels of milk intake, similar to those to be used in the experimental work and balancing the calcium levels with a readily available source such as calcium lactate, would be a valuable test. Five litters of albino mice were used, 1 animal of each litter was killed as a measure of the calcium content of the mice in its respective litter and 1 mouse of

TABLE 2

The effect of varying levels of intake of calcium and dried skim milk on the utilization of dietary calcium in weanling male albino mice.[1]

DIETS			FOOD INTAKE	GAIN IN WEIGHT		CALCIUM	
No.	Dried skim milk	Calcium		Total	Per gm of food	Increment	Utilization factor
	%	%	gm	gm	gm	mg	%
M-2	20	0.279	49.7	10.2	0.21	83	61 ± 3.1 [2]
M-3	15 [3]	0.281	51.3	10.6	0.20	83	58 ± 2.2
M-4	10 [4]	0.280	59.3 [5]	10.2 [5]	0.17 [5]	88	54 ± 2.0
M-5	25	0.343	50.1	9.2	0.19	86	51 ± 3.1
M-6	20 [3]	0.345	48.9	10.6	0.22	86	52 ± 2.5

[1] The values for diets M-2, M-3 and M-4 represent averages of 5 animals; those for diets M-5 and M-6, averages of 4 animals.

[2] Probable error of the mean.

[3] 0.5% of calcium lactate added.

[4] 1.0% of calcium lactate added.

[5] Data from 1 animal (G-3) that ate much less than the others and gained only 2.3 gm are excluded.

each litter was placed on each diet. Dried skim milk, at levels of 10, 15, 20 and 25% was fed and the calcium level of the diets containing 10 and 15% was balanced at those of 20 and 25% dried skim milk by adding the necessary amounts of calcium lactate as shown in table 2. The animals on the 10% skim milk plus calcium lactate made similar gains to those on the 20% dried skim milk but did not make quite as efficient use either of the total food or of the ingested calcium, showing an average gain per gm of food eaten of 0.17 gm and a utiliza-

tion of 54 ± 2.0% as compared with 0.21 gm and 61 ± 3.1%, respectively, for the mice on the 20% dried skim milk diet. This difference in utilization was only of a very slight statistical significance. We, therefore, felt justified in the use of 20 and 10% levels of dried skim milk, the levels commonly used in rat experiments of this type. It is desired to call attention to the slightly poorer utilization of calcium at the higher level of intake, 51 ± 3.1% at 0.343% calcium intake as against 61 ± 3.1% at the 0.279% level. While these differences are slight, it is believed that they are significant as is further shown in table 3.

TABLE 3

The utilization of dietary calcium by weanling male albino mice at various intake levels.[1]

DIETS			FOOD INTAKE	GAIN IN WEIGHT		CALCIUM	
No.	Dried skim milk	Calcium		Total	Per gm of food	Increment	Utilization factor
	%	%	gm	gm	gm	mg	%
M-5a	25	0.343	40.2	8.4	0.21	61	45 ± 1.1[2]
M-2a	20	0.280	43.6	10.4	0.24	80	66 ± 1.8
M-3b	15	0.222	42.9	9.7	0.23	74	77 ± 2.0
M-4b	10	0.172	41.4	9.3	0.22	61	86 ± 3.7

[1] All values are averages of results with 5 mice.

[2] Probable error of the mean.

The utilization of the calcium of dehydrated carrots and cabbage was tested at several levels of intake. In a preliminary experiment (not shown in the tables) with animals of 30 days of age instead of the usual 20 days, utilizations of 38 ± 0.7% for 6 mice from 3 litters on the 20% dried skim milk diet at a calcium level of 0.28% and for 7 mice on the carrot diet an average of 32 ± 1.2% at an average level of 0.22% calcium intake were obtained. Because of the low calcium content of the dried carrots a very high percentage of carrots (over 50% of the diet) was necessary to replace 10 gm of dried skim milk calcium. This high proportion of carrots in the diet was not well tolerated by the mice. Therefore, in subsequent experiments (table 4), various levels of the vege-

tables were used. With the cabbage 10, 7 and 4 gm of the dried skim milk and with the carrots 7 gm instead of the usual 10 gm were replaced by the vegetable calcium.

The results (table 4) with the carrots bore out the general idea of the preliminary experiment that calcium of carrots is not as well utilized as milk calcium. Statistically this difference (43 as compared with 58%) proved definitely significant and it is felt that for mice, carrots are a poorer source of calcium than is milk. McCluggage and Mendel ('18) with

TABLE 4

The utilization of dietary calcium of weanling male albino mice[1] on diets containing 20% dried skim milk compared to the utilization on similar diets in which from 20 to 50% of the milk calcium is replaced by the calcium of dehydrated cabbage or carrots.

DIETS			FOOD INTAKE	GAIN IN WEIGHT		CALCIUM	
No.	Dried skim milk	Dehydrated vegetables		Total	Per gm of food	Increment	Utilization factor
	%	%	gm	gm	gm	mg	%
M-2a	20		45.9	10.4	0.23	74	58 ± 1.0[2]
Cab-1	10	28[3]	53.3	10.0	0.19	66	45 ± 1.0
Cab-2	13	20[3]	56.5	11.3	0.20	75	48 ± 0.8
Cab-3	16	12[3]	55.1	12.1	0.22	77	50 ± 1.5
C-3	13	38[4]	49.1	8.6	0.17	58	54 ± 0.4

[1] All values are averages of 5 mice. All diets contained 0.28% calcium.
[2] Probable error of the mean.
[3] Cabbage.
[4] Carrots.

dogs, Shields, Fairbanks, Berryman and Mitchell ('40) with growing rats, and Breiter, Mills, Rutherford, Armstrong and Outhouse ('42) with adult humans, have also found this to be true. On the other hand, Rose ('20) and Edelstein ('32), working with adult humans and children, respectively, found carrots highly effective in maintaining calcium balances.

The generally lower utilization in the preliminary experiment of both milk (38 against 58%) and carrots (32 against 43%) is probably largely due to the differences in ages for as discussed later mice tend to show a poorer utilization of cal-

cium of cabbage as the age increases. The fact that the difference between the utilization of carrot and milk calcium in the preliminary experiment (32 against 38%) was much less than in the subsequent experiment of table 4 (43 against 58%) is due, at least in part, to the difference in the levels of dietary calcium, which as shown in table 3 significantly affects utilization values.

The dehydrated cabbage was fed at 3 levels of 12, 20 and 28% replacing 4, 7 and 10 gm of the 20 gm of skim milk, respectively. The utilization values of 50, 48, and 45% as against 58% for milk (table 4) show a definitely lower utilization of cabbage calcium. Also, as expected, a progressively lower utilization was observed as the per cent of milk replaced was increased. At the 13% dried skim milk level, the values of 43 $\pm$ 0.4% for carrots and the 48 $\pm$ 0.8% for cabbage show a slightly better utilization of cabbage calcium as compared to that of carrots. Kelly ('43) found that in the rat, Savoy cabbage calcium was 80% utilized as against 86% for milk, but fed both at relatively low levels of calcium intake.

A further attempt was made to investigate the apparent difference of utilization at various calcium levels noted previously (table 2). Four litters of mice were used as before except that 2 mice from each litter were killed as base line controls of initial calcium content. Four diets containing 10, 15, 20 and 25% dried skim milk were fed. The percentage utilization is inversely related to the calcium intake, as table 3 clearly shows.

A further attempt was made to check the previous observation that the age of the mice was an important factor in the percentage utilization of dietary calcium. As a few females were available, it was also decided to see if there were appreciable sex differences in utilization. Weanling mice from 2 litters (T and U) fed diet Cab-3 with a calcium content of 0.28% were used. The first group of males, T-2, T-3 and U-3, were killed after 12 days and showed utilization values of 61, 56 and 62%. The other males, T-4, and U-1, were killed after 24 days of feeding and showed values of 54 and 51% utiliza-

tion. Five males on this same diet (Cab-3) in a previous experiment (table 4) had shown an average value of 50 ± 1.5% over a 20-day period which is considered a good check between the 2 different experiments. The 2 females, T-1 and U-2, were killed after 24 days and showed slightly lower values of 46%.

TABLE 5

The calcium content of albino mice[1] *under various conditions.*

NO. OF MICE	SEX	AGE	TOTAL WEIGHT	BODY CALCIUM		REMARKS
		days	*gm*	*mg*	%	
5		0–1	5.3		0.292	newborn
2		15	10.3		0.577	sucklings
30	M	19–22			0.70 ± 0.008	weanlings
9	F	21–22			0.70 ± 0.017	weanlings
6	M	31			0.76 ± 0.009	1 week on 20% dried skim milk diet.
16	M	40			0.73 ± 0.007	3 weeks on 20% dried skim milk diet.
6	M	56			0.70 ± 0.005	5 weeks on 20% dried skim milk diet.
4(3F)		21			0.64 ± 0.007	weanlings of litter X
10(4F)		22			0.70 ± 0.006	weanlings of litter W
4	F	22			0.69 ± 0.007	weanling females of litter W
6	M	22			0.70 ± 0.007	weanling males of litter W
1	F	120	24.7	198.0	0.802	adult had just weaned 4 mice.
1	F	120	28.4	211.5	0.745	adult had weaned 6 mice 1 week before.
1	F	120	29.1	229.6	0.788	adult had weaned 7 mice 1 week before.
1	F	120	23.0	230.8	1.003	adult ate litter 3 weeks before.
1	F	120	23.9	239.7	1.002	adult ate litter 3 weeks before.
1	M	120	23.0	230.0	1.000	adult male.
1	M	120	24.7	249 6	1.010	adult male

[1] All animals after weaning had been on the basic 20% powdered skim milk diets (nos. M-2, M-2a).

Admittedly the number of animals used here is too small to show other than trends. However, the values are consistent and do bear out the observations in earlier experiments.

Table 5 includes data on the calcium content of albino mice at various ages. In general, the total content and percentage of calcium in the body increases from birth to adult life as Sherman and MacLeod ('25) and Briwa and Sherman ('41) found for rats. However, the data fail to show a significant sex difference for mice such as these authors found with rats.

SUMMARY AND CONCLUSIONS

The technique of the Sherman group for the determination of available calcium in vegetables using rats has been adapted to albino mice and, for our purposes, is considered to be superior as it results in a marked saving of space, time, materials and money in routine investigations of the availability of calcum of foodstuffs.

Litters of weanling albino mice at 20 days of age, in litter mate comparisons, were fed diets in which nearly all of the dietary calcium was derived from dried skim milk. The utilization values obtained were compared to those of similar diets in which up to one-half of the milk calcium was replaced by that of dehydrated vegetables, i.e., cabbage and carrots.

The mice utilized $45 \pm 1.0\%$ of the dietary calcium from the cabbage diet when one-half of the dried skim milk calcium was replaced by cabbage calcium as compared with a utilization of $58 \pm 1.0\%$ of the calcium from the control (20% dried skim milk) diet. When only 35% of the milk calcium was replaced by the vegetables tested, calcium utilizations of $48 \pm 0.8\%$ were obtained for the cabbage diet and $43 \pm 0.4\%$ for the carrot diet as compared with $58 \pm 1.0\%$ for the control diet.

The utilization of milk calcium was found to vary from $45 \pm 1.1\%$ at a 25% dried skim milk level (0.34% dietary calcium) to $86 \pm 3.7\%$ at a 10% skim milk level (0.17% dietary calcium).

There appeared to be a tendency for poorer utilization of dietary calcium as the age of the mice increased. A slightly poorer utilization by females was noted in the case of 2 females tested.

The data on total calcium content of albino mice showed values of about 0.3% at birth rising steadily to a little over 1% in adults, with no appreciable variation due to sex.

It was also apparent that weanling mice show a definitely lower utilization of dietary calcium than do weanling rats at a similar intake level.

LITERATURE CITED

ADOLPH, W. H., AND S. S. CHEN 1932 The utilization of calcium in soy bean diets. J. Nutrition, *5:* 379.

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1940 Methods of Analysis, 5th edition.

BASU, K. P., M. N. BASAK AND H. N. DE 1942 The availability of the calcium ingested in the process of chewing betel nut leaves with lime. Indian J. Med. Res., *30:* 309.

BASU, K. P., H. N. DE AND M. N. BASAK 1942 The bones of small fish as a source of nutritionally available calcium and phosphorous. Indian J. Med. Res., *30:* 417.

BENDAÑA-BROWN, A., AND F. GERONIMO 1941 Unpublished work. Manila.

BREITER, H., R. MILLS, J. DWIGHT, B. MCKEY, W. ARMSTRONG AND J. OUTHOUSE 1941 The utilization of the calcium of milk by adults. J. Nutrition, *21:* 351.

BREITER, H., R. MILLS, E. RUTHERFORD, W. ARMSTRONG AND J. OUTHOUSE 1942 The utilization of the calcium of carrots by adults. J. Nutrition, *23:* 1.

BRIWA, K. E., AND H. C. SHERMAN 1941 The calcium content of the normal growing boy at a given age. J. Nutrition, *21:* 155.

EDELSTEIN, E. 1932 Der Einfluss von Gemusezulagen auf den Stickstoff und Mineralstoffwechsel des Kindes. Z. Kinderheilk, *52:* 483.

FINCKE, M. L. 1941 The utilization of the calcium of cauliflower and broccoli. J. Nutrition, *22:* 477.

FINCKE, M. L., AND H. C. SHERMAN 1935 The availability of calcium from some typical foods. J. Biol. Chem., *110:* 421.

KAO, H. C., R. T. CONNER AND H. C. SHERMAN 1938 The availability of calcium from Chinese cabbage (Brassica pekinensis, Rupr.). J. Biol. Chem., *123:* 221.

KELLY, J. 1943 The availability of the calcium of some New Zealand vegetables. J. Nutrition, *25:* 303.

KUNG, L. C., H. L. YEH AND W. H. ADOLPH 1938 The availability of calcium in vegetable food materials. Chinese J. Physiol., *13:* 307.

LUNDE, G., AND J. LIE 1940 The utilization of calcium in fish and fish bones. Tidj. Kjemi Bergvesen, *20:* 16; C. A. *34:* 3788.

MCCLUGGAGE, H. B., AND L. B. MENDEL 1918 Experiments on the utilization of nitrogen, calcium, and magnesium in diets containing carrots and spinach. J. Biol. Chem., *35:* 353.

MACDONALD, A. J., AND S. BOSE 1942 The growth promoting value of eggs. Indian J. Med. Res., *30:* 285.

POTGIETER, M. 1940a The utilization of calcium and phosphorus of taro by young rats. J. Am. Diet. Assn., *16:* 670.

——— 1940b The utilization of calcium and phosphorus of taro by young women. J. Am. Diet. Assn., *16:* 898.

ROSE, M. S. 1920 Experiments on the utilization of the calcium of carrots by man. J. Biol. Chem., *41:* 349.

SHERMAN, H. C., AND F. L. MACLEOD 1925 The calcium content of the body in relation to age, growth, and food. J. Biol. Chem., *64:* 429.

SHIELDS, J. B., B. W. FAIRBANKS, G. H. BERRYMAN AND H. H. MITCHELL 1940 The utilization of calcium in carrots, lettuce, and string beans in comparison with the calcium in milk. J. Nutrition, *20:* 263.

STEGGERDA, F. R., AND H. H. MITCHELL 1946 Variability in the calcium metabolism and calcium requirements of adult human subjects. J. Nutrition, *31:* 407.

AMINO ACIDS IN THE URINE OF HUMAN SUBJECTS FED EGGS OR SOY BEANS [1]

BETTY F. STEELE, H. E. SAUBERLICH, MAY S. REYNOLDS AND C. A. BAUMANN

Departments of Biochemistry and Home Economics, College of Agriculture, University of Wisconsin, Madison

(Received for publication September 23, 1946)

INTRODUCTION

The presence of amino acids in human urine has been recognized for many years but there has been relatively little information on the individual acids, largely because no suitable analytical methods were available for the quantitative determination of small amounts of these substances in complex mixtures. In the present study diets of known composition were fed to human subjects, and the urine collected was analyzed for 16 amino acids by established microbiological techniques (Schweigert et al., '44; Greenhut et al., '46; McMahan and Snell, '44).

EXPERIMENTAL

The diets were part of a large series designed to reveal differences in the biological availability of protein from soy beans at different stages of maturity and prepared in various ways. The general procedure was the same as that used by Murlin and coworkers ('38). Four human subjects (college women) consumed diets in which 80% of the total nitrogen was supplied by mature dried soy beans or by eggs, the

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the Wisconsin Alumni Research Foundation and the Jonathan Bowman Cancer Fund.

"reference protein." The soy beans were prepared by soaking over night and boiling for 60 minutes, or by autoclaving at 110°C. for 30 minutes. The protein level of the diets supplied 5% of the total calories and the remaining calories were divided equally between carbohydrate and fat. Food furnishing approximately 45 cal. per kg of body weight was consumed daily. The daily intake of 3 of the 4 subjects is indicated in table 1. These subjects weighed an average of 64 kg; the fourth subject weighed only 56 kg and received 87% as much nitrogen as the others.

TABLE 1

Composition of diets consumed daily.

COMPONENT	DIET I	DIETS II AND III
	gm	*gm*
Egg	216	
Mature dried soy beans		77 [1]
Butter	49	59
Sucrose	114	101
Cream	107	107
Cornstarch	75	75
Crisco	30	30
Karo	28	28
Lettuce	100	100
Carrots	50	50
Grapefruit juice	200	200
Applesauce	150	150
Lactose	70	70
Salad oil	10	10
Gm of nitrogen	5.17	5.13

[1] Diet II contained boiled soy beans; the soy beans in diet III were autoclaved.

The subjects consumed the diets for periods of 6 days each in the following sequence: egg protein diet I; boiled mature soy protein diet II; egg protein diet I; autoclaved mature soy protein diet III. Routine determinations of nitrogen balance were made throughout each of the experimental periods. The urine samples analyzed for amino acids were those collected on the sixth day of each of the last 3 dietary periods. The specimens from individual subjects were preserved under

toluene and HCl, and the total daily urine was diluted to 1500 ml, adjusted to a pH of 7, and then 15 ml were further diluted to 100 ml for the microbiological assays. Amino acids (tables 2–4) were then determined with *Lactobacillus arabinosus*, *Leuconostoc mesenteroides*, or *Streptococcus faecalis R* by methods previously employed on urine from experimental animals (Sauberlich and Baumann, '46).

RESULTS

The subjects were in good health on all diets. They maintained their weights and remained in nitrogen equilibrium for the second and third experimental periods although they went into negative nitrogen balance during the fourth period when autoclaved soy protein was fed (Steele, '46). An average of 5.13 gm of nitrogen was consumed daily during the latter period, while the average loss of nitrogen per day was 5.77 gm (5.31–6.26).

Fourteen of the 16 amino acids determined were found in most of the specimens of urine analyzed (tables 2 to 4). Leucine was always absent from the urine when the egg protein or the boiled soy bean protein was fed, but on the autoclaved soy bean diet, on which the subjects were in negative nitrogen balance, some leucine appeared in the urine of all subjects. Aspartic acid was never present in microbiologically available form in any of the specimens of urine. Occasional samples of urine were also devoid of isoleucine, lysine, or proline, although these latter amino acids were usually present and the average excretion of proline was one of the highest of all acids when expressed as the percentage of that ingested appearing in the urine. There were no significant variations in the excretion of amino acids by the different subjects nor did there appear to be any consistent relationship between the amounts of the different amino acids ingested and the amounts excreted in the "free" form in the urine. The most abundant amino acids in the egg diet were leucine, aspartic acid, and glutamic acid, and 2 of these did not appear in the urine as

TABLE 2

Urinary excretion of amino acids by 4 human subjects ingesting egg protein.

AMINO ACIDS INGESTED DAILY [1]		AMINO ACIDS EXCRETED DAILY		PERCENTAGE OF INGESTED AMINO ACIDS EXCRETED	
		Range	Average	Range	Average
	gm	*mg*	*mg*	*%*	*%*
Arginine	1.99	3.5– 5.0	4.4	0.17– 0.26	0.22
Aspartic acid [2]	3.38	0.0	0.0	0.0	0.0
Cystine	0.57	70.7–81.3	76.2	11.78–16.12	13.40
Glutamic acid [1]	3.32	30.0–93.0	61.0	0.86– 2.79	1.84
Histidine	1.12	51.4–84.0	74.6	4.44– 8.30	6.68
Isoleucine	1.59	0.0– 5.0	5.0	0.00– 0.30	0.31
Leucine	5.37	0.0–	0.0	0.00	0.00
Lysine	1.79	0.0– 6.0	1.5	0.00– 0.32	0.08
Methionine	0.98	0.8– 4.3	2.4	0.10– 0.52	0.25
Phenylalanine	1.69	3.2– 8.0	4.9	0.18– 0.45	0.29
Proline [1]	0.92	0.0–20.0	7.0	0.00– 2.08	0.51
Serine [2]	2.88	13.0–33.0	27.0	0.44– 1.16	0.95
Threonine	1.42	21.0–27.0	24.9	1.68– 1.82	1.75
Tryptophane	0.48	5.8– 8.2	7.5	1.16– 1.83	1.56
Tyrosine	1.51	13.0–25.0	16.5	0.82– 1.91	1.10
Valine	1.35	4.4–11.5	7.3	0.31– 0.82	0.55

[1] Average ingestion for the 4 subjects. The amounts of amino acids ingested daily were calculated from the percentages of amino acids in eggs as published in Block and Bolling ('45), except those marked [1] which are from Jacobs, M., Food and Food Products, vol. 1 ('44) and those marked [2] which were determined in this laboratory.

"free"[2] acids, while only 61 mg of the 3324 mg of glutamic acid ingested daily was excreted free in the urine. The most abundant amino acids in the urine were cystine and histidine, neither of which was particularly abundant in any of the diets fed.

[2] The term "free" amino acid is used to indicate those forms of the acid available to the species of microorganism used in the assay. This would include the amino acids in certain peptides, such as leucyl glycine which can satisfy the needs of the organism for these amino acids (Kuiken et al., '43), although the amino acids of many other peptides do not appear to be microbiologically available (Lewis and Olcott, '45).

Expressed as the percentage of ingested amino acid excreted in microbiologically available form, wide differences were observed between the different amino acids. Thus on the egg diet the percentage of ingested leucine and aspartic acid excreted was 0.0% and it was less than 0.5% for arginine, isoleucine, lysine, methionine, and phenylalanine. On the other hand, 13.4% of the ingested cystine appeared in the urine and 6.8% of the ingested histidine. These latter 2 amino acids were also the most abundant in the urines excreted on the soy bean diets (tables 3 and 4).

TABLE 3

Urinary excretion of amino acids by 4 human subjects ingesting protein of boiled soy beans.

AMINO ACIDS INGESTED DAILY [1]		AMINO ACIDS EXCRETED DAILY		PERCENTAGE OF INGESTED AMINO ACIDS EXCRETED	
		Range	Average	Range	Average
	gm	*mg*	*mg*	%	%
Arginine	1.67	4.5– 5.5	5.1	0.23– 0.37	0.36
Aspartic acid [3]	3.98	0.0	0.0	0.00–	0.00
Cystine	0.20	66.6–72.9	68.7	31.80–37.20	34.00
Glutamic acid [2]	4.32	25.0–44.0	33.2	0.56– 1.16	0.76
Histidine	1.06	55.9–87.5	73.2	5.14– 8.10	6.92
Isoleucine	1.42	0.0–10.0	4.5	0.00– 0.68	0.32
Leucine	2.05	0.0– 0.0	0.0	0.00	0.00
Lysine	1.62	0.0– 6.0	3.0	0.00– 0.41	0.37
Methionine	0.63	0.7– 4.5	.2.5	0.12– 0.71	0.40
Phenylalanine	1.73	4.0–11.8	7.1	0.22– 0.66	0.41
Proline [1]	0.99	0.0–20.0	7.0	0.00– 2.30	0.60
Serine [2]	1.51	13.0–26.0	21.3	0.89– 1.66	1.41
Threonine	1.18	15.8–22.0	19.7	1.29– 1.80	1.67
Tryptophane	0.48	5.0– 9.0	7.5	1.16– 1.97	1.55
Tyrosine	1.26	8.0–14.0	11.0	0.61– 1.08	0.88
Valine	1.29	3.1– 5.0	4.1	0.23– 0.38	0.32

[1] Average ingestion for the 4 subjects. The amounts of amino acids ingested daily were calculated from the percentages of amino acids in eggs as published in Block and Bolling ('45), except those marked (1) which are from Jacobs, M., Food and Food Products, vol. 1 ('44), (2) Baumgarten et al. ('46), and those marked (3) which were determined in this laboratory.

When boiled soy beans were fed, the excretion of "free" amino acids was very similar qualitatively to that observed on the diet containing eggs, in spite of the different amino acid mixture that was present (tables 3 and 4). The soy bean diet contained more glutamic acid than the egg diet and presumably also more nitrogenous compounds other than the 16 amino acids determined, since the percentages of total nitrogen were essentially equal on both diets. The soy bean diet contained less leucine, less methionine, and appreciably less serine and cystine than the egg diet. Nevertheless the amounts of cystine excreted were essentially the same whether eggs or soy beans were fed. Since the daily intake of cystine was relatively low on the soy bean diets, the percentages of excretion appeared to be particularly high on the latter, 34.4 and 36.6% on boiled and autoclaved soy beans, respectively, as contrasted to 13.4% on eggs. With the single exception of leucine, there were no very marked differences between the excretion of amino acids during the periods representing the 2 kinds of soy beans (tables 3 and 4). No leucine appeared when the boiled soy bean diet was eaten, while 13.0 mg were excreted daily in the urine when autoclaved soy beans were fed. But if, as suggested above, the appearance of leucine on the latter diet was related to the negative nitrogen balance of the subjects, the mechanism for the excretion of leucine would still appear to differ from that for the other amino acids, since the excretion of the other acids was not altered greatly by the negative nitrogen balance (tables 3 and 4). Leucine has been reported to be present in certain pathological urines, presumably combined with tyrosine (Hawk and Bergeim, '44).

Significantly there were no essential differences in the excretion of methionine on the 3 diets, although methionine is apparently the limiting amino acid in raw soy beans, and the degree of availability depends on the heat treatment applied. Nor did the excretion of lysine appear to be in any respect unusual in these experiments. Lysine is relatively labile to heat (Greaves, Morgan and Loveen, '38) and a measurable

destruction of lysine has been reported in over-heated soy bean meal (Clandinin et al., '46). In the present experiments the daily average excretion of lysine was 1.5 mg during the autoclaved soy bean period and 3.0 mg on boiled soy beans. This

TABLE 4

Urinary excretion of amino acids by 4 human subjects ingesting protein of autoclaved soy beans.

AMINO ACIDS INGESTED DAILY [1]		AMINO ACIDS EXCRETED DAILY		PERCENTAGE OF INGESTED AMINO ACIDS EXCRETED	
		Range	Average	Range	Average
	gm	mg	mg	%	%
Arginine	1.67	6.0–10.0	8.1	0.35– 0.68	0.49
Aspartic acid [3]	3.98	0.0	0.0	0.0	0.0
Cystine	0.20	62.9–91.7	74.0	30.00–43.50	36.60
Glutamic acid [2]	4.32	23.0–70.0	49.0	0.51– 1.83	1.13
Histidine	1.06	61.8–77.0	69.3	5.70– 7.10	6.55
Isoleucine	1.42	2.0–12.0	4.5	0.14– 0.81	0.32
Leucine	2.05	6.0–23.0	13.0	0.28– 1.24	0.64
Lysine	1.62	0.0– 6.0	1.5	0.00– 0.41	0.10
Methionine	0.63	1.2–10.0	4.3	0.10– 1.54	0.68
Phenylalanine	1.73	2.0– 4.1	3.2	0.11– 0.27	0.19
Proline [1]	0.99	0.0–20.0	15.0	0.00– 2.30	2.02
Serine [2]	1.51	21.0–38.0	26.5	1.34– 2.78	1.75
Threonine	1.18	21.0–23.0	22.0	1.70– 2.18	1.87
Tryptophane	0.48	6.6–11.0	8.4	1.30– 2.20	1.74
Tyrosine	1.26	13.0–17.0	14.2	1.00– 1.30	1.09
Valine	1.29	6.0–11.5	9.2	0.45– 1.00	0.72

[1] Average ingestion for the 4 subjects. The amounts of amino acids ingested daily were calculated from the percentages of amino acids in eggs as published in Block and Bolling ('45), except those marked [(1)] which are from Jacobs, M., Food and Food Products, vol. 1 ('44), [(2)] Baumgarten et al. ('46), and those marked [(3)] which were determined in this laboratory.

difference might suggest a difference in lysine intake. On both diets, however, variations within groups were wide and in-individual samples of urine were encountered in which no lysine was present. Nevertheless, if the differences in individual excretion between the 2 groups should prove to be

significant, it would appear that boiling soy beans for 60 minutes was less deleterious to lysine than autoclaving at a higher temperature for a shorter period of time. It is even possible that the negative nitrogen balance of the subjects on the autoclaved beans may have been due to an insufficiency of available lysine. Murlin and co-workers ('46) added lysine to a baked soy bean diet and observed a higher biological value as tested on human subjects.

Peptides

Experiments on urine from rats and mice (Sauberlich and Baumann, '46) suggest that at least half of the amino acids in mouse urine is present as peptides, while in rat urine the percentage of such bound amino acids exceeds 75%. Preliminary experiments in the present study suggest that bound amino acids were also present in the samples of human urine analyzed. The urine was hydrolyzed in 2 N acid or 5 N $Ba(OH)_2$ for 5 hours at 15 pounds pressure and the samples were then re-assayed for certain of the amino acids. No leucine was found, although recoveries of leucine added prior to hydrolysis ranged from 97 to 133%. Hence it was concluded that this acid is not present in normal human urine in either the free form nor as a peptide. However, the amounts of microbiologically available tyrosine, tryptophane, and serine were found to have increased 4 to 5 fold after hydrolysis. A 24-hour specimen of urine which contained no aspartic acid before hydrolysis contained 88 mg of this acid after the peptides were hydrolyzed. Aspartic acid, therefore, is an example of an amino acid that exists in human urine almost entirely in a bound form. Several other workers have concluded that human urine contains peptides (Van Slyke, '13; and Albanese et al., '46).

DISCUSSION

In a previous study it was observed (a) that the amounts of the amino acids excreted in the urine by rats and mice de-

pended upon the amounts ingested, (b) that the percentages of ingested acid appearing in the urine in microbiologically available form were approximately the same for all amino acids except cystine and (c) that all of the 16 amino acids determined were present in every specimen of rat or mouse urine examined (Sauberlich and Baumann, '46).

The present results on human urine indicate that the excretion of amino acids by man differs from that by the rat or mouse in each of these respects. The amounts of any one amino acid excreted by human subjects were essentially the same whether eggs or boiled soy beans were fed, in spite of differences in the amino acid content of the two diets. Many specimens of human urine were encountered that lacked one or more of the amino acids proline, lysine, or isoleucine; free aspartic acid was never present, and leucine only under special circumstances. On the other hand histidine and cystine were always present in relatively high percentages of the amounts ingested.

These differences between species suggest that in general the human organism exerts more control than rats or mice over the excretion of amino acids into the urine, and furthermore that not all amino acids are controlled to the same extent. It is also possible that deamination reactions proceed more rapidly or completely in man than in the rat or mouse.

Folin and Berglund ('22) observed that the amounts of alpha amino nitrogen in the urine of men and dogs increased with increases in the blood, and concluded that there is no renal threshold for amino acids comparable to that for glucose. The colorimetric method of analysis employed (Folin, '22), however, lacks specificity, and does not measure all amino acids to the same extent. It is evident from the present data that "thresholds" for the amino acids do exist and that they vary from one amino acid to another. Presumably amino acids differ in the ease with which they can be reabsorbed from the glomerular filtrate through the renal tubules. Leucine is an example of an amino acid which must be very efficiently reabsorbed, since it does not appear in human urine, although

human blood contains from 17.3 to 25.7 μg per ml of plasma (Hier and Bergeim, '46). These amounts do not differ significantly from those for other essential amino acids in blood, including histidine, which occurs in urine in relatively large amounts.

The high urinary excretion of cystine observed requires some comment. In the previous study (Sauberlich and Baumann, '46) more cystine was found in the urine of rats and mice than the amounts of any other amino acid, but this result was not emphasized because the diet contained cystine in the free form while all other amino acids were present only as protein (casein). In the present experiments with human subjects, however, the cystine fed was all combined as protein, and again cystine was the most abundant amino acid in the urine. Cystine can function in the detoxication of many aromatic compounds (Stekol, '46) whence it appears in urine combined as mercapturic acids. Cystine was also one of the first free amino acids to be detected in urine (Alsberg and Folin, '05) and an abnormally high excretion of this amino acid has been noted more frequently than that of any other. It is possible, however, that microbiological methods of determination will reveal metabolic abnormalities similar to cystinuria but involving other acids. Polarographic determinations (Reed, '42) indicated an excretion of 40 to 80 mg of cystine — cysteine daily in urine from normal human males; in the present study daily microbiological values ranged from 60 to 91 mg. The latter method likewise measures both cystine and cysteine.

SUMMARY

1. Diets containing eggs or soy beans as sources of protein were fed to 4 human subjects at a protein level corresponding to 5% of the ingested calories, and the amino acid content of the urine was determined microbiologically.

2. Cystine was invariably found to be excreted in the highest percentage of the amount ingested, 12 to 44%. Histidine was next, 4 to 8%. The percentage excretion for most amino acids was found to range from about 0.2 to 1.8% of that ingested.

Proline, lysine, and isoleucine were frequently absent from the specimens of urine analyzed, free aspartic acid was always absent, while leucine was present only when the subjects were in negative balance (autoclaved soy bean diet).

3. There were no group differences between the excretion of essential and non-essential amino acids.

4. The hydrolysis of urine resulted in appreciable increases in microbiologically available tyrosine, serine, tryptophane, and aspartic acid. Apparently many amino acids in human urine were bound as peptides.

LITERATURE CITED

ALBANESE, A. A., L. E. HOLT, J. E. FRANKSTON AND V. IRBY 1946 Estimation and characterization of bound amino nitrogen of normal human urine. Fed. Proc., *5:* 118.

ALSBERG, C., AND O. FOLIN 1905 Protein metabolism in cystinuria. Am. J. Physiol., *14:* 54.

BAUMGARTEN, W., A. MATHER AND L. STONE 1946 Essential amino acid composition of feed materials. Cereal Chem., *23:* 135.

BLOCK, R., AND D. BOLLING 1945 Amino Acid Composition of Protein and Foods. Charles C. Thomas, Springfield, Ill.

CLANDININ, D. R., W. W. CRAVENS, C. A. ELVEHJEM AND J. G. HALPIN 1946 Deficiencies in over-heated soy bean oil meal. Abstracted Poultry Sci., *25:* 399.

FOLIN, O. 1922 A new colorimetric method for the determination of the amino acid nitrogen in blood. J. Biol. Chem., *51:* 377.

FOLIN, O., AND H. BERGLUND 1922 The retention and distribution of amino acids with especial reference to the urea formation. J. Biol. Chem., *51:* 395.

GREAVES, E. O., A. F. MORGAN AND M. K. LOVEEN 1938 Effect of amino acid supplements and of variations in temperature and duration of heating upon the biological value of heated casein. J. Nutrition, *16:* 115.

GREENHUT, I. T., B. S. SCHWEIGERT AND C. A. ELVEHJEM 1946 The amino acid requirement of *Streptococcus faecalis* and the use of this organism for the determination of threonine in natural products. J. Biol. Chem., *162:* 69.

HAWK, P. B., AND O. BERGEIM 1944 Practical Physiological Chemistry. The Blakiston Company, Philadelphia, Pa.

HIER, S. W., AND O. BERGEIM 1946 The microbiological determination of certain free amino acids in human and dog plasma. J. Biol. Chem., *163:* 129.

JACOBS, M. 1944 Food and Food Products, *1.* Interscience Publishers, Inc., New York.

KUIKEN, K. A., W. H. NORMAN, C. M. LYMAN, F. HALE AND L. BLOTTER 1943 The microbiological determination of amino acids. I. Valine, leucine, and isoleucine. J. Biol. Chem., *151:* 615.

LEWIS, J. C., AND H. S. OLCOTT 1945 A lactobacillus assay method for l(+) glutamic acid. J. Biol. Chem., *157:* 265.

MCMAHAN, J. R., AND E. E. SNELL 1944 The microbiological determination of amino acids. I. Valine and arginine. J. Biol. Chem., *152:* 83.

MURLIN, J. R., E. S. NASSET AND M. E. MARSH 1938 The egg replacement value of the proteins of cereal breakfast foods, with a consideration of heat injury. J. Nutrition, *16:* 249.

MURLIN, J. R., L. E. EDWARDS, E. E. HAWLEY AND L. C. CLARK 1946 Biological value of protein in relation to the essential amino acids which they contain. II. Interconvertibility of biological values illustrated by supplementing egg and soy protein with essential amino acids. J. Nutrition, *31:* 555.

REED, G. 1942 The polarographic estimation of cystine in urine. J. Biol. Chem., *142:* 61.

SAUBERLICH, H. E., AND C. A. BAUMANN 1946 The effect of dietary proteins upon amino acid excretion by rats and mice. J. Biol. Chem., *166:* 417.

SCHWEIGERT, B. S., J. M. MCINTIRE, C. A. ELVEHJEM AND F. M. STRONG 1944 The direct determination of valine and leucine in fresh animal tissues. J. Biol. Chem., *155:* 183.

STEELE, B. F. 1946 The digestibility and biological value of Mendota soy bean protein with some values for urinary amino acid excretion. M. S. Thesis, University of Wisconsin Library, Madison.

STEKOL, J. A. 1946 Studies on the mercapturic acid synthesis in animals. XIV. On the synthesis of mercapturic acids in man. J. Biol. Chem., *164:* 651.

VAN SLYKE, D. D. 1913 Improved methods in the gasometric determination of free and conjugated amino acid nitrogen in the urine. J. Biol. Chem., *16:* 125.

THE BIOCHEMICAL DEFECT UNDERLYING THE NUTRITIONAL FAILURE OF YOUNG RATS ON DIETS CONTAINING EXCESSIVE QUANTITIES OF LACTOSE OR GALACTOSE

PHILIP HANDLER

Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina

(Received for publication September 23, 1946)

The inadequacy of galactose as the carbohydrate of rat dietaries has been reported by Guha ('31), and of lactose by Ershoff and Deuel ('44), and Handler and Dubin ('46). Young rats, fed a diet in which lactose or galactose is the sole carbohydrate, fail to grow properly and die in 3–20 days. The present report describes several attempts which have been made to determine the physiological mechanism underlying this nutritional failure.

EXPERIMENTAL

All rats were males of the Vanderbilt strain (Wolfe, Bryan and Wright, '38). They were grown to a weight of 45 gm on a commercial stock chow after weaning, then housed in individual cages and offered the experimental diets. The basal diet (in %) consisted of casein 10, lactose (or other carbohydrate) 81, salts (Hubbell, Mendel and Wakeman, '37) 4, cod liver oil 2, cottonseed oil 3, corn oil 1. In addition each kilo of diet contained thiamine 3 mg, riboflavin 5 mg, pyridoxine 3 mg, calcium pantothenate 30 mg, nicotinic acid 5 mg, choline chloride 3 gm, inositol 2 gm, *p*-aminobenzoic acid 0.5 gm, mixed tocopherols 50 mg, and naphthohydroquinone acetate 50 mg.

Various modifications of this diet were fed to groups of 10 rats each. The results are summarized in table 1. It will be seen that galactose, or an equal mixture of glucose and galactose was as deleterious in its effects as lactose itself. This is in agreement with the work of Guha ('31) but in contrast to the findings of Ershoff and Deuel ('44). It would seem to indicate that the galactose moiety of the lactose molecule is responsible for the observed effects. Increasing the protein

TABLE 1

The effects of dietary modifications on lactose toxicity.

DIET	DIETARY MODIFICATION	WEIGHT CHANGE	SURVIVAL TIME
		gm	*days*
A	Sucrose 81%	23.0	14.0 [1]
B	Lactose 81% — diarrhea	— 19.1	4.9
	no diarrhea	— 12.7	7.3
C	Galactose 81% — diarrhea	— 18.3	5.2
	no diarrhea	— 15.6	6.8
D	Galactose 81% Glucose 40%	— 15.3	7.9
E	Lactose 60%	— 9.4	10.7
F	Lactose 40%	7.1	14.0 [1]
G	Galactose 20% Glucose 61%	6.8	... [2]
H	Casein 30% Lactose 61%	0.8	11.2
J	Lard 20% Lactose 61%	3.9	12.3
K	Sulfasuxidine 3% Lactose 78%	— 15.9	7.2
L	Cellulose 10% Lactose 71%	— 13.3	7.7
M	$NaHCO_3$ 0.5% Lactose 80.5%	— 16.1	6.8
N	Saline drinking water Lactose 81%	— 14.0	7.0

[1] Animals sacrificed after 2 weeks.

[2] Two rats died on eleventh day, remainder sacrificed after 2 weeks.

and/or fat content of the diet did increase the rats' survival time and at sufficiently high levels afforded complete protection. However, this does not seem necessarily to have been due to any specific ameliorating effect of the protein or fat but, rather, may perhaps be attributed to the diminished lactose ingestion. The critical level of lactose in the diet appeared to be about 60–70% while that of galactose was 30–40%. In agreement with Ershoff and Deuel ('44) and Guha ('31) it was found that when rats weighing more than

125 gm were offered the high lactose or galactose diets, they grew very slowly but survived for several months, developing the lenticular opacities typical of such diets, as indeed did many of the young rats before they died.

Since most rats showed at least a moderate diarrhea when fed the lactose diets (and to a lesser extent on the galactose diets as well) attempts were made to control this with both cellulose and sulfasuxidine. However, neither of these did effectively control the diarrhea nor did they appreciably lengthen the rats' survival time. Because of the possibility of dehydration and acidosis due to both the diarrhea and absorption of lactic acid from the bowel, one group was offered 0.4% NaCl in place of drinking water while 0.5% of $NaHCO_3$

TABLE 2

Blood constituents after lactose feeding.

DIETARY CARBOHYDRATE	SERUM PROTEINS	CO_2 CAPACITY	LACTIC ACID	PHOSPHATASE	CA	P	NPN
	gm %	*vol. %*	*mg %*	*B U.*[1]	*mg %*		*mg %*
Sucrose	7.3	56	22	16.6	10.1	7.4	31
Lactose	6.9	52	25	31.0	12.4	6.3	44
Galactose	6.7	57	20	37.3	11.8	5.0	41

[1] Bodansky Units.

was added to the ration of another group. Again, neither of these measures increased the rats' survival time and actually, as will be seen later, no serious acidosis or dehydration did occur in lactose or galactose-fed animals.

Blood was obtained by decapitation of a number of moribund animals on the lactose and galactose diets and from controls on the sucrose diet. Analyses were performed for serum proteins, CO_2 combining power, lactic acid, phosphatase, calcium, phosphorus and non-protein nitrogen. The results are summarized in table 2. Each value represents the mean of a group of 12 rats although in most instances blood from a number of rats was pooled in order to obtain a quantity sufficient to perform the determination. From the hematocrit,

serum proteins and alkali reserve it was adjudged that no serious dehydration or acidosis resulted from either lactose or galactose feeding. While lactose may stimulate the development of an acidophilic intestinal flora the lactic acid was either lost in the stool or readily handled by the liver as its concentration did not rise in the systemic circulation nor did it provoke an acidosis. It is doubtful that the slightly elevated non-protein nitrogen values were of primary importance in the death of lactose or galactose-fed animals but represent a terminal event. The elevation of serum calcium is compatible with the known enhanced absorption of calcium from the intestine produced by lactose feeding while the decreased

TABLE 3

Urinary constituents during lactose feeding. Mean 24-hr. values per rat during third week of feeding. Initial rat weight 150 gm.

DIETARY CARBOHYDRATE	VOLUME	pH	TITRATABLE ACIDITY	NH_3	SUGAR	ACETONE BODIES	CA	P
	ml		*ml 0.1 N NaOH*		*gm*		*mg*	*mg*
Sucrose	14	6.5	1.8	1.2	0	0	1.4	1.6
Lactose	43	5.9	5.7	6.4	0.48	0	9.2	1.9
Galactose	68	5.6	5.9	7.8	1.40	0	5.7	3.8

phosphorus may be associated either with the elevated serum calcium concentration or with the disturbed carbohydrate metabolism to be discussed later. A discussion of the increased serum alkaline phosphatase will be reserved for discussion in a later report (Handler, Follis and Baylin, in preparation).

Adolescent rats weighing 150 gm initially were placed in metabolism cages and urine was collected from pairs of rats. Three pairs of rats were placed on each of diets A, B and C. A 24-hour urine was collected from each pair every fourth day for 60 days. The urines were preserved with alcoholic thymol. The results are summarized in table 3. Each value is the mean of 18 samples collected from 3 pairs of rats.

The increased urine volume observed on the lactose and galactose diets was the result of the quite considerable excretion of reducing sugar seen in these animals. The values expressed in the table were calculated as galactose, and indeed, since acid hydrolysis failed to increase the reducing power of the urine from lactose-fed rats it would seem that these animals excreted no lactose. Osazones prepared from the urines of lactose and galactose-fed rats appeared identical in crystal structure with an authentic sample of galactosazone and gave no depression of the melting point in mixed melting point determinations. However, in no case was the sugar excretion of sufficient magnitude to impose a serious caloric want. This was also true in young animals as well.

A tendency toward acidosis was certainly indicated by the acid urines and high excretion of titratable acid and ammonia by rats on both the lactose and galactose diets. While this might be thought to be due at least in part to the diarrhea, these acid urines were also encountered from rats producing reasonably well-formed stools and so it seems not unlikely that the tendency to acidosis was occasioned by the production and excretion of some unidentified organic acid other than lactic, pyruvic, acetoacetic or β-hydroxybutyric. Since no diminution in the alkaline reserve was found, the renal excretion of this acid adequately prevented a systemic acidosis.

The markedly increased excretion of calcium by both lactose and galactose-fed rats is in keeping with the known enhanced intestinal absorption of calcium produced by lactose ingestion. Since complete balance studies were not performed it is not possible to state whether there occurred any unusually great retention of calcium or even, perhaps, actual bone demineralization.

Complete autopsies were performed on 4 rats fed galactose diet C and 4 fed lactose diet B. The animals were sacrificed when moribund, the tissues fixed in 10% neutral formalin, and sections were stained with hematoxylin and eosin. Heart, lungs, liver, spleen, pancreas, kidney, adrenal, stomach, intestine, brain, thyroid and bone were examined. However,

nothing was found which seemed contributory to an understanding of the disease process. The intestines were greatly distended with gas and contained heavy bacterial colonies. No intussusception, volvulus, or ulceration was noted. The lymphoid tissues, particularly spleen, all appeared atrophic as they do in general inanition. Blood samples removed from such animals under sterile conditions failed to reveal any bacteremia. Despite the unusual turnover of calcium and the somewhat elevated serum calcium concentrations, no evidence of calcification of soft tissues, blood vessels or kidney was found. The latter is also in keeping with the essentially normal blood NPN values. X-ray examination revealed only a complete failure of skeletal calcification in adolescent rats maintained on lactose or galactose. This will be reported and discussed in detail in a subsequent publication (Handler, Follis and Baylin).

Bonnamour and Escallon ('13) found a diminished calcium and increased phosphorus content in the femur of a rabbit which had been given 10 gm of lactose intravenously daily for 3 months. While it seemed unlikely that this sort of process, if real, could have accounted for the large urinary calcium excretions found in the present animals, it was thought of interest to study the effects of intraperitoneally administered glucose, sucrose, lactose, galactose and β-lactose. Each of these was given as 10 ml of a 5% solution daily to groups of 3 rats whose initial weight was 150 gm each and which were maintained on the basal sucrose-containing diet A. Twice each week for 2 months, 24-hour urines were collected and calcium, phosphorus and sugar excretion determined. No differences were noted in the various groups and, other than the urine volumes and sugar excretion, they did not differ essentially from a control group given 10 ml of isotonic saline per day. At the end of the experimental period the animals were sacrificed, their femurs removed, soaked in 95% ethyl alcohol for 24 hours and then in ether for 12 hours. They were then dried in an oven, weighed, digested with hot 0.1 N HCl for 2 hours, made to volume after which aliquots were taken for

calcium and phosphorus analyses. While the femurs of the lactose, β-lactose and galactose injected rats weighed slightly less than those given glucose, sucrose or saline, the calcium and phosphorus concentrations and the Ca/P ratios were not significantly different. These results are not in agreement with the report of Bonnamour and Escallon ('13) on a rabbit, and under these conditions, intraperitoneal administration of these various carbohydrates for 2 months did not produce any significant decalcification of rat femurs.

TABLE 4

Carbohydrate metabolism on lactose and galactose diets.
(Each value mean of 6 rats)

INITIAL RAT WEIGHT	TIME	DIETARY CARBOHYDRATE	BLOOD GLUCOSE	BLOOD GALACTOSE	SERUM P	LIVER GLYCOGEN
gm	*days*		*mg %*	*mg %*	*mg %*	*%*
50	4	Sucrose	112	...	6.5	3.4
50	4	Lactose	89	106	5.4	1.7
50	4	Galactose	78	285	4.8	2.5
50	7	Sucrose	122	...	7.4	3.5
50	7	Lactose	67	320	6.0	0.7
50	7	Galactose	48	473	4.6	0.4
150	10	Sucrose	97	...	8.3	3.0
150	10	Lactose	83	98	6.9	1.3
150	10	Galactose	87	316	5.1	3.1

Carbohydrate metabolism studies were performed in the following manner. Blood and liver samples were obtained from sacrificed young rats after 4 days on diets B (lactose) and C (galactose) and from other rats on these diets when they appeared moribund (6–9 days). Control animals receiving diet A were sacrificed after 4 and 7 days. Samples were also obtained from 150-gm rats after they had been on diets A, B, and C for 10 days. Total reducing sugar, "true glucose" (Somogyi, '27) and phosphorus analyses were performed on the blood samples, and glycogen was determined in the liver (Good, Kramer and Somoygi, '33). The results are summarized in table 4.

Both lactose and galactose feeding consistently resulted in a lowering of the blood true glucose, the serum phosphorus and liver glycogen in young rats. The effect of galactose on the serum phosphorus was much more marked than was that of lactose. Liver glycogen values were actually lower in the lactose than in galactose-fed adolescent rats and the young ones sacrificed after 4 days but not in moribund young rats. This may perhaps have been due to the poorer state of these animals occasioned by the severe diarrhea. Probably the most significant finding was the reduced true glucose values obtained in the moribund rats on both lactose and galactose diets.

DISCUSSION

From the data presented herein and the studies of Ershoff and Deuel ('44) and Guha ('31) there is little doubt but that high concentrations of lactose in the diet are toxic to the rat. However, the physiological mechanism by which this toxicity is exerted has not yet been completely resolved. From the present work and that of Guha it would appear that it is the galactose moiety of the lactose molecule which is responsible for the observed effects. This, however, is contraindicated by the fact that while both lactose and β-lactose proved toxic in the diets of rats of the Long-Evans and the U.S.C. strains, equal amounts of galactose resulted only in a temporary weight loss followed by slow growth (Ershoff and Deuel, '44). Nevertheless, all indications in the present work seem to implicate galactose per se as the toxic factor.

Ershoff and Deuel ('44) concluded that the fatal effects of dietary lactose were probably dependent upon events in the intestine. It does not seem reasonable that, by changing the intestinal flora, lactose removed or diminished the supply of some, as yet unknown, dietary factor so essential to the animal economy that death resulted in as little as 3 days. Apparently not considered by previous workers was the possibility of death due to diarrhea with consequent acidosis and dehydration. However, this too has been ruled out since

blood studies showed no evidence of acidosis or dehydration nor did saline drinking water or $NaHCO_3$ prove therapeutically effective. Moreover, many rats, particularly those on the galactose diets, died in a few days yet showed reasonably well-formed stools during this period.

The effects of dietary lactose on calcium metabolism also seemed a possible etiologic factor in the death of these animals. Lactose, by stimulating the development of an acidophilic intestinal flora (Rettger and Cheplin, '23; Hudson and Parr, '24) lowers the pH of the intestinal tract below the duodenum (Robinson and Duncan, '31). It may be this phenomenon which is responsible for the enhanced intestinal absorption of calcium (Robinson and Duncan, '31). It seemed possible that there might exist a rational correlation between our observation that young rats die while adults survive indefinitely on lactose diets and the finding of French and Cowgill ('37), that in both rats and dogs lactose promotes calcium absorption in young animals but not in adults. While the markedly increased urinary excretion of calcium and slightly elevated serum calcium concentrations were compatible with the aforementioned effects of lactose, there were no indications that these changes were of any significance in the pathogenesis of this disease. Further, death appeared to be due to the presence of galactose, and the effects of galactose on calcium metabolism observed in the present work were not as marked as those of lactose.

Within 24 hours after being offered a lactose-containing diet virtually all rats showed the gross symptomatology described by Ershoff and Deuel ('44). They exhibited ruffled fur, edema of the hind paws, occasionally alopecia and were all generally filthy in appearance. This would all appear to be referable to the diarrhea and was only seen in those rats receiving galactose which also were afflicted with diarrhea. However, no animal actually appeared moribund, weak and listless on either lactose or galactose diets until there occurred a fall in the blood glucose concentration (table 4).

Guha ('31) concluded that the fatal results of galactose feeding were due to starvation. This was based upon the known slow utilization of galactose, its relatively low renal threshold and the increased appetite of rats on a high galactose diet. It is not possible to completely deny this possibility, but it does not seem likely when one considers that galactose-fed rats, eating as much as 7 gm a day excreted no more than 2.2 gm of galactose in the urine. If the remaining dietary galactose had been utilized properly such rats should survive almost indefinitely although their growth rate would be expected to be below normal. Instead they died in approximately the same time as did rats permitted access only to water. The possibility that death is due to simple starvation is also compatible with the observations of Holt and Kajdi ('44) that starving rats offered only lactose or galactose died as rapidly as did the unsupplemented controls while glucose, fructose, sucrose and various reasonably purified fats prolonged the rats' survival time as much as 25 days. Nevertheless, the extremely rapid course of the disease, the lack of any specific histological findings, the numbers of animals which died even more rapidly than do rats suffering from simple inanition, and the rather unexpected findings of Holt and Kajdi ('44) with respect to lactose and galactose described above, all point to the probability that death was due to some fundamental metabolic disturbance, presumably of carbohydrate metabolism. The findings in the present work suggest that not only was there a failure to utilize fully and properly the dietary lactose and galactose but that, perhaps, these actually interfered with normal carbohydrate (glycogen or glucose) metabolism. The studies reported herein do not permit any definitive statement of the nature of this disturbance but do indicate that galactose in the amounts given here may interfere with normal glucose metabolism. Thus, despite the presence of blood galactose concentrations as high as 600 mg %, moribund rats on lactose and galactose diets were found to have blood glucose concentrations as low as 40 mg % while the supply of liver glycogen appeared to be virtually ex-

hausted. It may be that this is related to the observation that liver glycogen after galactose feeding is built of 18 hexose units rather than the 12 units found in liver glycogen after glucose feeding (Bell, '36).

This situation appears to be analogous to that in infants showing galactosemia and cataracts (Bruck and Rapoport, '45; Goldbloom and Brickman, '46). In these children also were found high blood galactose levels and relatively low blood glucose concentrations as long as they were maintained on milk. When additional galactose was administered in tolerance tests, the blood galactose concentrations were elevated still further while the blood glucose fell to levels as low as 40 mg %. It should be noted that the blood glucose of normal children also fell to as low as 50 mg % during galactose tolerance tests. However, the blood glucose returned to normal and the blood galactose disappeared considerably more rapidly in the normal children than in the infants with "idiopathic galactosemia." Further, the clinical signs and symptoms of this state all appeared to be referable to the disturbance in carbohydrate metabolism and disappeared when the patients were placed on a lactose-free regime.

From consideration of the behavior of the rats described in the present report and the response of normal children to galactose tolerance tests it appears that the galactosemia with its untoward sequelae observed in some children as long as their diets contain lactose (as milk) is not really a qualitative idiopathic disturbance but rather a somewhat exaggerated quantitative response similar to that which can be obtained in normal rats and children if the galactose intake is sufficiently great.

SUMMARY

Weanling rats of the Vanderbilt strain die within 3 to 17 days after being placed on diets containing more than 60% lactose or 40% galactose. No lesions other than those associated with simple inanition were found by histological examination of their tissues. While most rats exhibited a

marked diarrhea and all a profound diuresis, death did not appear to have been due to dehydration or acidosis. While all rats had a marked calcinuria due to the enhanced calcium absorption from the intestine, their serum calcium concentrations were only moderately elevated and did not appear to be an etiologic factor in the death of such rats. Moribund rats on both lactose and galactose diets were found to have blood galactose concentrations varying from 210 to 640 mg % with true blood glucose levels which varied from 26 to 73 mg %. Simultaneously there occurred an appreciable drop in the serum inorganic phosphorus concentration and almost complete depletion of the liver glycogen. It is concluded that this disturbance of carbohydrate metabolism, which is similar to that occasionally observed in infants while they drink milk, is the prime etiologic factor in the death of rats on such diets. The occasional "galactosemia" seen in infants is thought to represent not a qualitative idiopathic phenomenon but a quantitative exaggeration of events which can be elicited in a normal animal when the galactose intake is sufficiently high. The mechanism whereby galactose interferes with normal glucose metabolism has not yet been clearly defined.

ACKNOWLEDGMENTS

The author's thanks are due to the Nutrition Foundation and the Duke University Research Council for their support of this work; to Merck and Company, Inc., for a supply of the crystalline B vitamins used in this study; and to Drs. R. H. Follis, Jr. and T. N. Lide who very kindly performed the histological studies reported herein.

LITERATURE CITED

BELL, D. J. 1936 Liver glycogen IV. Molecular structure of glycogen formed after ingestion of galactose by the fasting rabbit. Biochem. J., *30:* 1612.

BONNAMOUR, A. B., AND ESCALLON 1913 Décalcification et lésions osseuses chez le lapin sous l'influence du lactose en injections intraveineuses. Compt. rend. soc. biol., *74:* 1106.

BRUCK, E., AND S. RAPOPORT 1945 Galactosemia in an infant with cataracts. Amer. J. Dis. Child., *70:* 267.

ERSHOFF, B. H., AND H. J. DEUEL, JR. 1944 Inadequacy of lactose and β-lactose as dietary carbohydrates for the rat. J. Nutrition, *28:* 225.

FRENCH, R. B., AND G. R. COWGILL 1937 Immaturity of the organism as a factor determining the favorable influence of lactose on the utilization of calcium and phosphorus. J. Nutrition, *14:* 40.

GOLDBLOOM, A., AND H. F. BRICKMAN 1946 Galactemia. J. Pediatrics, *28:* 674.

GOOD, C. A., H. KRAMER AND M. SOMOGYI 1933 The determination of glycogen. J. Biol. Chem., *100:* 485.

GUHA, B. C. 1931 On galactose as the dietary carbohydrate. Biochem. J., *25:* 1385.

HANDLER, P., AND I. N. DUBIN 1946 The significance of fatty infiltration in the development of hepatic cirrhosis due to choline deficiency. J. Nutrition, *31:* 141.

HANDLER, P., R. H. FOLLIS, JR. AND G. J. BAYLIN The effects of caloric restriction on calcium and phosphorus metabolism. (In preparation.)

HOLT, L. E., JR., AND C. N. KAJDI 1944 The nutritional requirements in inanition I., Observations on the ability of single foodstuffs to prolong survival. Bull. Johns Hopkins Hosp., *74:* 121.

HUBBELL, R. G., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, *14:* 273.

HUDSON, N. P., AND L. W. PARR 1924 Relation of reaction of intestinal contents to diet and flora. J. Infect. Dis., *34:* 621.

RETTGER, L. F., AND H. A. CHEPLIN 1923 A treatise on the transformation of the intestinal flora with special reference to the implantation of Bacillus acidophilus. Yale Univ. Press, New Haven.

ROBINSON, C. S., AND E. W. DUNCAN 1931 The effect of lactose and the acid-base value of the diet on the hydrogen ion concentration of the intestinal contents of the rat and their possible influence on calcium absorption. J. Biol. Chem., *92:* 435.

SOMOGYI, M. 1927 Reducing non-sugars and true sugars in human blood. J. Biol. Chem., *75:* 33.

WOLFE, J. M., W. R. BRYAN AND A. W. WRIGHT 1938 Histologic observations on the anterior pituitaries of old rats with particular reference to spontaneous appearance of pituitary adenomata. Am. J. Cancer, *34:* 352.

EFFECT OF COOKING AND CURING ON LYSINE CONTENT OF PORK LUNCHEON MEAT

O. H. M. WILDER AND H. R. KRAYBILL
Research Laboratory, American Meat Institute,
The University of Chicago, Illinois

THREE FIGURES

(Received for publication August 26, 1946)

The well-known reaction between nitrous acid and primary amines has led to the question of whether the "nitrite cure" impairs the lysine value of pork. It has been suggested that lysine would be among the first of the amino acids to be attacked if there were any reactions between individual amino acids in the proteins of meat, and the nitrite used in the curing process. In organic chemistry, reactions between primary amines and nitrous acid are ordinarily carried out in strongly acid solutions. The pH of meat never reaches such high acid values, but the high temperature reached during the heat treatment — about 112° to 113°C. — might favor the reaction.

Heat alone, particularly dry heat or toasting, has been shown to have an injurious effect on many proteins. Invaldsen ('29), Daniel and McCollum ('31), Morgan ('31), Maynard and Tunison ('32), Schneider ('32), Maynard, Bender and McCay ('32), Fixen and Jackson ('32), Fairbanks and Mitchell ('35), Murlin, Nasset and Walsh ('38), Stewart, Hensley and Peters ('43), Block, Cannon et al. ('46) and others have demonstrated that dry heat or toasting has an injurious effect on many proteins; and Greaves, Morgan and Loveen ('38) and Block, Cannon et al. ('46) have shown that the lysine in the protein molecule is particularly sensitive to

relatively mild processing procedures. In the cooking of meat, the temperature would probably not go as high as in a toasting process, but Morgan and Kern ('34) have reported that cooking by 3 different processes tended to lower the biological value of beef.

The commercial canning of luncheon meat involves both a nitrite cure and cooking, both of which might have some effect on the lysine content of the protein. If they have any effect on lysine, it may not be a destruction since Block, Jones and Gersdorff ('34) have reported that as much lysine could be isolated after acid hydrolysis of heated casein as from unheated, and Seegers and Mattill ('35) found that acid hydrolysis of heat impaired proteins restored their nutritive value when tryptophane, alone, was added.

The paucity of knowledge at the present time regarding the interactions of lysine in the protein molecule makes it difficult to interpret results secured by any one method of analysis. Animal feeding trials are still the ultimate tests for the availability of any one essential amino acid. Accordingly, animal feeding trials were conducted to get information on the relative nutritive values of fresh and processed pork luncheon meat, and to determine if there is any destruction of lysine during the cooking or curing process.

EXPERIMENTAL PROCEDURE

A representative batch of ground pork was obtained from a batch prepared for commercial production of canned pork luncheon meat. It was divided into 3 parts: one part was frozen as fresh meat, a second part was canned, and the third part was cured by a standard commercial method and then canned. Both of the canned lots were heat processed at the temperature and pressure used commercially in the packing plant. The curing ingredients included 0.15 pounds of sodium nitrite per 1000 pounds of meat. After curing and remixing, the meat was stuffed into 12-ounce cans and closed under vacuum in a commercial closing machine. The cooked samples

were heated in a retort for 1 hour at a temperature of 112° to 113°C., and then immediately cooled with cold water.

The meats were all stored in a refrigerator, the fresh meats being kept frozen until they were fed to the animals. When needed, a can was removed and opened, the meat ground twice through a small meat grinder, and mixed.

The 3 lots of meat were fed to young weanling rats as supplements to a basal ration composed of (in %) ground yellow corn 85.6, hydrogenated vegetable oil 9.5, salt mixture 4, sardine oil 0.5, l(−) cystine 0.2, and l(−) tryptophane 0.2 parts, respectively. In addition, each rat received 0.2 ml daily of a commercial preparation [1] as a source of the B vitamins. Previous (unpublished) feeding trials have shown that the greatest deficiency in this ration is with regard to the lysine content, and that young rats consuming the ration will grow but only very slowly. However, when the ration is supplemented with 0.2% l(+) lysine, growth is very markedly increased. It was calculated that when the rats were allowed 10 gm of the basal ration daily, 50% or more of the animals' requirements for amino acids were met, with the exception of the lysine requirement, where only about 20% of the requirement was supplied. Lysine, then, was the first limiting factor.

Ten rats were maintained on this diet throughout the experiment as negative controls. The other rats were divided into groups of 10 animals each, and given a daily supplement of 0.5 or 1.0 gm of the 3 meats, respectively, fresh, cooked, or cured-cooked. The basal ration was limited to 10 gm daily, and fed in the regular feed cup. The vitamin B supplement was pipetted into a small dish daily, and the meats were weighed out daily for each individual rat and dropped into the feed cup. Daily feed consumption records and weekly rat weight records were kept for a period of 8 weeks.

One can of meat from each of the three groups was dried, defatted, and analyzed for lysine by the specific decarboxylase method of Hanke ('46).

[1] Vitab.

DISCUSSION OF RESULTS

Growth curves showing gain in weight of the rats are presented in figures 1, 2 and 3. The rats on the basal ration alone gained very slowly, averaging only 22 gm in 8 weeks. When this same ration was supplemented with 0.2% of l(+) lysine, growth was increased to 82.5 gm in 8 weeks (0.2% l(+) lysine in the basal ration is equivalent to 20 mg of lysine when the rats are allowed 10 gm of food daily). The depression of the rate of gain in weight observed by Mitchell and Smutts ('32) when tryptophane is added to a corn ration does not appear to be a factor when lysine is also added.

When the meats were fed at a level of 0.5 gm daily as a supplement to the basal ration, growth was substantially increased over that observed in animals receiving no supplementary meat or lysine (figs. 1 and 2), and a further increase was observed in the rats fed 1.0 gm of the meats daily (fig. 3).

The addition of the meats to the rat diets added considerable amounts of other animo acids that were in short supply in the basal diet, but the increases in growth were in no case as high as was observed when the basal ration was supplemented with 0.2% of l(+) lysine. The amount of lysine added to the rats' daily diet through the meats (table 1) was in no case as high as the 20 mg consumed daily by the control group, hence, the limiting factor for growth was still lysine when these low levels of meat were fed.

Figure 1 presents the gain in weight obtained by feeding 0.5 gm of the meats daily. While the growth observed on the cured-cooked meat is slightly less than on the other meats, it is not regarded as significant since in another series of rats fed the same rations (fig. 2) growth on the cured-cooked meat was as high as that observed on the cooked, and higher than that observed on the fresh meat. The growth observed on rats fed the cooked meats, in general, runs slightly higher than any of the others, even when 1.0 gm daily was fed. This may be due in part at least, to the protein content of the meat. In the cooking process, a certain amount of water and fat separated from the uncured meat, and proved to be very difficult

to remix into the meat when the cans were opened. Some of the water was sometimes lost, which could account for the variation in the protein percentage from can to can. The fresh meat ranged from 15.6 to 16.2% protein, the cooked meat from 16.6 to 18.8, and the cured-cooked meat from 15.3 to 15.8% protein.

Figure 3, showing the gain in weight of rats receiving 1.0 gm of meat daily, indicates that the lysine even in this amount of meat is inadequate to support maximum growth, hence, if

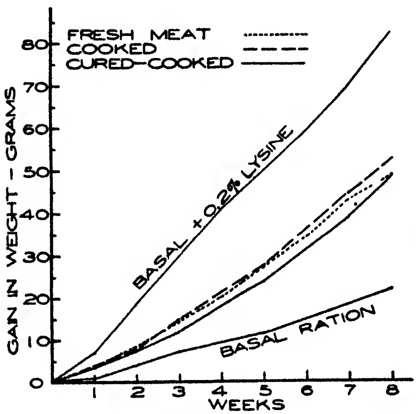


Figure 1

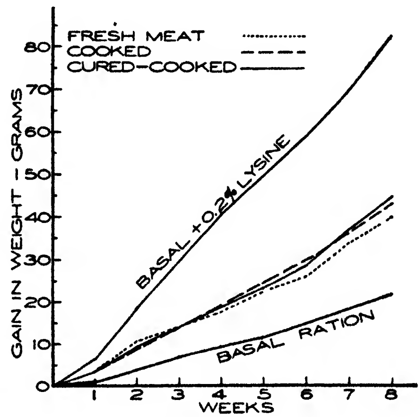


Figure 2

Fig. 1 Curves showing growth of rats receiving the lysine deficient basal ration, and daily supplements of 0.5 gm fresh (frozen), cooked, or cured-cooked pork luncheon meat, and 0.2% l(+) lysine (first series).

Fig. 2 Curves showing growth of a second series of rats fed the same supplements as in figure 1.

there were any significant differences in the lysine content of the 3 meats, it should appear in the growth curves of the rats receiving that amount or less of meat.

The lysine analysis of the 3 meats (table 1) indicates that there was some destruction of lysine in the cured-cooked meat but not in the cooked. When calculated to a 16% nitrogen basis, the difference between the lysine in the fresh (7.97%) and the cooked (8.06%) is not significant. The difference between the cured-cooked (7.01%) and the other two probably is significant, the loss of lysine being about 12%.

When the amounts of lysine are calculated, that each animal received per day in its meat supplement, the results can be correlated with the gains in weight shown in figure 1, but

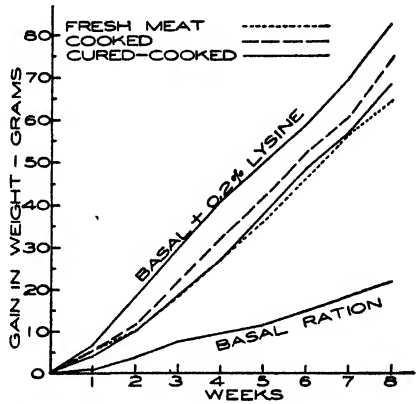


Fig. 3 Curves showing growth of rats receiving the lysine deficient basal ration, and daily supplements of 1.0 gm of fresh (frozen), cooked, or cured-cooked pork luncheon meat in comparison with 0.2% l(+) lysine.

TABLE 1

Lysine analysis of meats.

	FRESH MEAT	COOKED MEAT	CURED-COOKED MEAT
Lysine in meat calculated to 16% nitrogen basis — %	7.97	8.06	7.01
Average protein content of meat (N × 6.25) — %	15.9	17.7	15.5
Average lysine in meat as fed — %	1.267	1.426	1.086
Lysine consumed per day, 0.5 gm. Meat level — mg	6.33	7.13	5.43
Lysine consumed per day, 1.0 gm. Meat level — mg	12.67	14.26	10.86

not so easily with the weight gains of other animals shown in figures 2 and 3.

Since the gains in weight observed in the rats on the 3 samples of meat are so nearly uniform at any one level of

meat fed, it seems highly improbable that there could have been any destruction of lysine greater than the 12% shown by the analyses. Insofar as the animal is concerned, the destruction may have been either insignificant at that level, or the destruction may have been less in other cans of the same meats that were opened and fed to the animals.

SUMMARY AND CONCLUSIONS

Pork luncheon meats were analyzed for lysine, and fed to rats as a supplement to a diet which was deficient in lysine.

Lysine analyses of fresh, cooked, and cured-cooked pork luncheon meat indicate that there was no destruction of lysine due to the cooking, but the cured-cooked sample showed a loss of 12% of the original lysine content.

No significant differences in growth were observed on rats fed the 3 samples of meat, indicating that if there was a destruction of lysine, it was too small to detect in the feeding tests with rats.

LITERATURE CITED

BLOCK, R. J., P. R. CANNON, R. W. WISSLER, C. H. STEFFEE, JR., R. L. STRAUBE, L. E. FRAZIER AND R. L. WOOLRIDGE 1946 The effects of baking and toasting on the nutritional value of proteins. Arch. Biochem., *10:* 295.

BLOCK, R. J., D. B. JONES AND C. E. F. GERSDORFF 1934 The effect of dry heat and dilute alkali on the lysine content of casein. J. Biol. Chem., *105:* 667.

DANIEL, E. P., AND E. V. MCCOLLUM 1931 Studies on the nutritive value of fish meals. U. S. Dept. Com., Bur. Fisheries Invest. Rept. 2, vol. 1.

FAIRBANKS, B. W., AND H. H. MITCHELL 1935 The nutritive value of skim-milk powders with special reference to the sensitivity of milk proteins to heat. J. Agr. Research, *51:* 1107.

FIXEN, M. A. B., AND H. M. JACKSON 1932 The biological values of proteins. IV. The biological values of wheat, maize and milk. Biochem. J., *26:* 1923.

GREAVES, E. O., A. F. MORGAN AND M. K. LOVEEN 1938 Effect of amino acid supplements and of variations in temperature and duration of heating upon the biological value of heated casein. J. Nutrition, *16:* 115.

HANKE, MARTIN E. 1946 Precise estimation of lysine in the Van Slyke-Neill manometric apparatus with a specific decarboxylase. Fed. Proc., *5:* 137.

INGVALDSEN, T. 1929 Fish meals. Part 1. The effect of the high temperature employed for drying on the nitrogen partition in fish meals. Canad. Chem. and Metall., *13:* 97.

MAYNARD, L. A., R. C. BENDER AND C. M. MCCAY 1932 Vitamin A and protein content of various fish meals. J. Agr. Res., *44:* 591.

MAYNARD, L. A., AND A. V. TUNISON 1932 Influence of drying temperature upon digestibility and biological value of fish proteins. Indus. and Eng. Chem., *24:* 1168.

MITCHELL, H. H., AND D. B. SMUTTS 1932 The amino acid deficiencies of beef, corn, oats and soybeans for growth in the white rat. J. Biol. Chem., *95:* 263.

MORGAN, A. F. 1931 The effect of heat upon the biological value of cereal proteins and casein. J. Biol. Chem., *90:* 771.

MORGAN, A. F., AND GRACE E. KERN 1934 The effect of heat upon the biological value of meat protein. J. Nutrition, *7:* 367.

MURLIN, J. R., E. E. NASSET AND M. E. WALSH 1938 Egg replacement value of the proteins of cereal breakfast foods, with consideration of heat injury. J. Nutrition, *16:* 249.

SCHNEIDER, B. H. 1932 Nitrogen balance studies with various fish meals. J. Agr. Research, *44:* 723.

SEEGERS, W. H., AND H. A. MATTILL 1935 The effect of heat and hot alcohol on liver proteins. J. Biol. Chem., *110:* 531.

STEWART, R. A., G. W. HENSLEY AND F. N. PETERS, JR. 1943 The nutritive value of protein. I. The effect of processing on oat protein. J. Nutrition, *26:* 519.

THE INFLUENCE OF PTEROYLGLUTAMIC ACID (A MEMBER OF THE VITAMIN M GROUP) ON THE ABSORPTION OF VITAMIN A AND CAROTENE BY PATIENTS WITH SPRUE[1]

WILLIAM J. DARBY, MARGARET M. KASER AND EDGAR JONES

Departments of Medicine and Biochemistry, Vanderbilt University School of Medicine, Nashville; and Tennessee Department of Public Health, Nashville

ONE FIGURE

(Received for publication September 24, 1946)

In diseases such as sprue, celiac disease, and cystic fibrosis of the pancreas, in which there is an impairment of the absorption of fat from the gastrointestinal tract, serum levels of carotene and vitamin A may be decreased. Also, the rise in serum vitamin A following the oral administration of a test dose of vitamin A may be absent or delayed (Chesney and McCoord, '33–'34; Clausen and McCoord, '38; Breese and McCoord, '39; May and McCreary, '41; May, McCreary and Blackfan, '42; Ingelfinger, '43; Adlersberg and Sobotka, '43; Cayer, Ruffin and Perlzweig, '45). Other findings which suggest a defective absorption of fat soluble factors in sprue are a hypoprothrombinemia (Fanconi, '38; Butt and Snell, '41; and Ingelfinger, '43) and a lowering of serum calcium, which may be due in part to a failure to absorb vitamin D (Bennett, Hunter and Vaughan, '32; Hanes, '43; Ingelfinger, '43). More recently it has been found that plasma concentrations of the

[1] This work was supported by grants from the Nutrition Foundation, Inc., the International Health Division of The Rockefeller Foundation, and the Tennessee Department of Public Health.

other fat-soluble vitamin, tocopherol, is lowered in steatorrheas (Darby, Cherrington and Ruffin, '46).

The many similarities of sprue in man and of vitamin M deficiency in the monkey led to the now well-confirmed demonstration that pteroylglutamic acid (PGA) is active in producing hematologic and clinical remissions in sprue (Darby and Jones, '45; Moore, Bierbaum, Welch and Wright, '45; Darby, Jones and Johnson, '46a, b; Spies, Lopez, Menendez, Minnich and Koch, '46; Spies, Milanes, Menendez, Koch and Minnich, '46). We have reported that oral glucose tolerance curves and vitamin A absorption curves became more nearly normal in sprue after treatment with PGA (Darby and Jones, '45; Darby, Jones and Johnson, '46a, b). At the time of these earlier reports sufficient time had not elapsed to evaluate with finality the effectiveness of this vitamin on the absorption of fat and fat-soluble factors. The data now indicate that treatment with PGA results in a definite decrease in the total fat loss in the feces of patients with sprue (Darby and Jones, unpublished observations). It is of importance, therefore, to determine whether there also occurs evidence of improved absorption of fat-soluble vitamin A in the cases of sprue treated with PGA.

EXPERIMENTAL

The subjects were 4 patients with sprue observed in the Vanderbilt University Hospital. Certain data on 3 of the cases were previously reported (Darby, Jones and Johnson, '46a, b). After diagnostic studies were completed the patients were treated with doses of either 5 or 15 mg daily of pteroylglutamic acid [2] administered either orally or intramuscularly. In 2 cases (J.D. and P.S.) therapy was discontinued after 14 and 19 days, in the others it has been continuous. One of the former patients has relapsed following the withdrawal of therapy, and a second remission has now been

[2] We are indebted to Dr. Stanton M. Hardy, Medical Director, Lederle Laboratories, Inc., for generous supplies of synthetic pteroylglutamic acid used in these studies.

induced by treatment with PGA. None of the patients have received supplements of carotene or vitamin A, and there has been no effort to alter their usual diets.

TABLE 1

The effect of pteroylglutamic acid (PGA) on serum carotene and vitamin A levels in sprue.

PATIENT	DATE	CAROTENE	VITAMIN A	REMARKS
		μg/100 ml	*I U./100 ml*	
J.D.	9-25-45	8	98	Before therapy
	9-29-45	18	95	Before therapy
	10- 4-45			Therapy instituted, 15 mg PGA daily, parenterally
	10-19-45	21	129	Remission
	10-22-45			Therapy discontinued
	11-21-45	22	133	Remission
	1-31-46	52	153	Remission
	3-20-46	72	158	Remission
	6-19-46	10	90	In relapse
	7-13-46	14		In relapse
	7-23-46	17	66	
	7-25-46			Therapy instituted, 5 mg PGA daily by mouth
	8-26-46	54	170	In remission
P.S.	10-18-45	12	90	Before therapy
	10-19-45			Therapy instituted, 15 mg PGA daily, parenterally
	10-22-45	19	60	
	11- 8-45			Therapy discontinued
	1- 3-46	63	66	Remission
P.B.	11- 9-45	21	119	Chronic sprue, before therapy
	11-10-45			Therapy instituted, 15 mg PGA daily, parenterally
	11-21-45	25	101	Remission
	12- 6-45	13	89	Remission
	7-17-46	65	124	Remission
W.O.	5- 6-46			Therapy instituted, 5 mg daily, orally
	5-16-46	36	133	Early remission
	5-25-46	73	103	Remission
	7-13-46	51	95	Remission

Serum carotene was estimated by measuring the absorption of a petroleum ether extract in an Evelyn photoelectric colorimeter at 440 μ and vitamin A was determined by means of the Carr-Price reaction as described by Kaser and Stekol ('43). Vitamin A tolerance tests were carried out by estimating the serum levels at intervals after the oral administration of 200,000 I.U. of vitamin A as a fish liver oil concentrate.

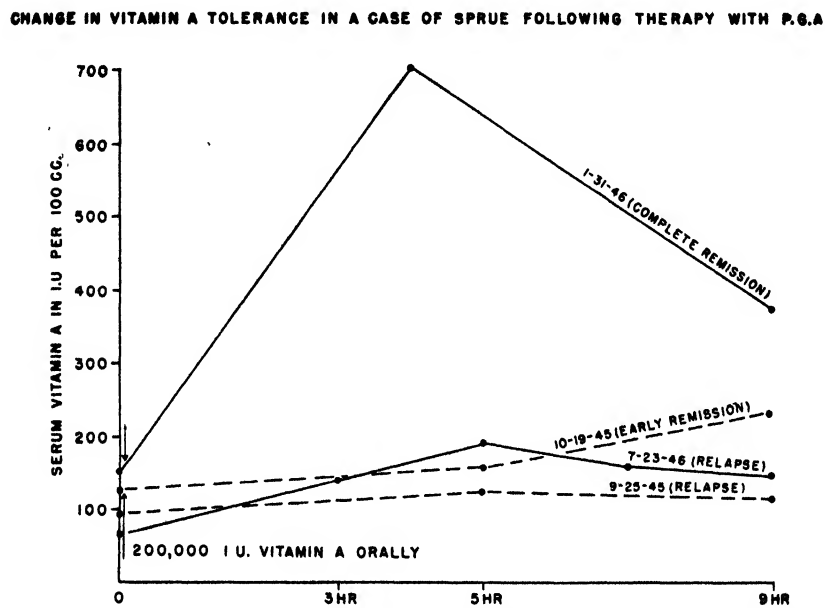


Fig. 1 Serum vitamin A levels following an oral dose of 200,000 I.U. of vitamin A to a patient with sprue. The slight increases observed on 3 occasions during relapse or early remission are in sharp contrast to the high rise observed during a complete remission following therapy with PGA.

RESULTS

The values for serum carotene and vitamin A are listed in table 1. Presumably the presence of adequate liver stores of vitamin A before the onset of sprue accounts for the satisfactory levels of vitamin A as found in these patients. All 4 subjects showed initial pronounced decreases in the serum carotene levels and a gradual increase in the carotene concen-

tration followed therapy with PGA. In one case (J.D.) these changes recurred during a second relapse and remission. It should be noted that as J.D. relapsed the carotene, which had approached a satisfactory level, again almost disappeared from his blood. There was also a simultaneous drop in tocopherol levels of the serum in this patient (Darby, Cherrington and Ruffin, '46).

Figure 1 depicts the vitamin A absorption curves for J.D. before treatment, during his clinical remission, and during the subsequent relapse. The profound change in the vitamin A absorption curves following therapy with PGA is apparent.

DISCUSSION

These results, together with those previously reported, indicate that a deficiency in man of the vitamin-M group, of which PGA is a member, results in defects of gastrointestinal absorption which may be reversed by supplying the deficient vitamin. The early cessation of the diarrhea, the reduction of the fat content of the stools, the return of the flat glucose tolerance curve to a normal one, and the rapid gain in weight in the patient with sprue treated with PGA are all evidences of improved absorption. In view of these changes the more slowly occurring increases in serum carotene concentration and the return of the vitamin A tolerance curve to normal during remission are consistent with the interpretation that PGA also favorably influences the absorption of carotene and vitamin A in sprue. The slower rate of correction of the absorption of fat-soluble substances than of water-soluble substances following the treatment of sprue with PGA is consistent with the more gradual disappearance of the steatorrhea in sprue after therapy with liver (Hanes, '43) and with *L. casei* factor (Darby, Jones and Johnson, '46b).

This evidence that the administration of one synthetic vitamin (PGA) alone may so greatly influence the concentration of another vitamin within the body may be cited as another example of an interrelationship of foodstuffs (Mitchell, '43; Moore, '45). It is impressive that a deficiency of vitamin M, as

seen in clinical sprue, results in demonstrable malabsorption of so many dietary factors — carbohydrate, fat, vitamins, and calcium. Additional experimental studies would seem to be indicated in order to determine whether any of the secondary deficiencies observed in the sulfa-treated ("vitamin M deficient") rat (Daft and Sebrell, '45) are due to impaired absorption. The gastrointestinal defects reported by McCarrison ('21) in his "vitamin B deficient" monkeys have been interpreted as due to a deficiency of thiamine (Williams and Spies, '38). It is now held that McCarrison's monkeys presented the clinical picture of vitamin M deficiency (Day, '44). It appears, therefore, that a reinvestigation of the relative roles of thiamine and of the members of the vitamin M group in maintaining gastrointestinal function would be timely.

SUMMARY

Serum carotene and vitamin A levels in 4 cases of sprue treated with pteroylglutamic acid (PGA) are reported. It was found that the low serum carotene levels observed in relapse were gradually increased following therapy. The carotene returned to its previous low level during the relapse of one patient following withholding of PGA, and again rose after a second period of therapy with PGA.

Following therapy with PGA an improved ability to absorb vitamin A is indicated by a rise in serum vitamin A following the administration of an oral test dose. The fact that this curve reverted to a typical flat curve during a relapse after the withdrawal of treatment is further evidence of the influence of this factor on the absorption of fat-soluble substances.

It is suggested that the M group of vitamins plays an important role in the normal physiology of the gastrointestinal tract.

LITERATURE CITED

ADLERSBERG, D., AND H. SOBOTKA 1943 Influence of lecithin feeding on fat and vitamin A absorption in man. J. Nutrition, *25:* 255.

BENNETT, T. I., D. HUNTER AND J. M. VAUGHAN 1932 Idiopathic steatorrhea (Gee's disease); a nutritional disturbance associated with tetany, osteomalacia and anaemia. Quart. J. Med., *1:* 603.

BREESE, B. B., JR., AND A. B. MCCOORD 1939 Vitamin A absorption in celiac disease. J. Pediatrics, *15:* 183.

BUTT, H. R., AND A. M. SNELL 1941 Vitamin K. W. B. Saunders Company, Philadelphia, p. 101.

CAYER, D., J. M. RUFFIN AND W. A. PERLZWEIG 1945 Vitamin levels in sprue. Am. J. Med. Sci., *210:* 200.

CHESNEY, J., AND A. B. MCCOORD 1933–34 Vitamin A of serum following administration of haliver oil in normal children and in chronic steatorrhea. Proc. Soc. Exp. Biol. Med., *31:* 887.

CLAUSEN, S. W., AND A. B. MCCOORD 1938 The carotinoids and vitamin A of the blood. J. Pediatrics, *13:* 635.

DAFT, F. S., AND W. H. SEBRELL 1945 Sulfonamides and vitamin deficiencies. Vitamins and Hormones, *3:* 49.

DARBY, W. J., M. E. CHERRINGTON AND J. M. RUFFIN 1946 Serum tocopherol levels in sprue. Proc. Soc. Exp. Biol. Med., *63:* 310.

DARBY, W. J., AND E. JONES 1945 Treatment of sprue with synthetic L. casei factor (folic acid, vitamin M). Proc. Soc. Exp. Biol. Med., *60:* 259.

DARBY, W. J., E. JONES AND H. C. JOHNSON 1946a The use of synthetic *L. casei* factor in the treatment of sprue. Science, *103:* 108.

——— 1946b Effect of synthetic Lactobacillus casei factor in treatment of sprue. J. Am. Med. Assn., *130:* 780.

DAY, P. L. 1944 The nutritional requirements of primates other than man. Vitamins and Hormones, *2:* 71.

FANCONI, G. 1938 Zoeliakie. Deut. Med. Wchnschr., *64:* 1565.

HANES, F. M. 1943 Sprue Tice's Practice of Medicine, W. F. Prior Company, Inc., Hagerstown, Md., *IV:* 171.

INGELFINGER, F. J. 1943 The diagnosis of sprue in nontropical areas. New Eng. J. Med., *228:* 180.

KASER, M. M., AND J. A. STEKOL 1943 A critical study of the Carr-Price reaction for the determination of β-carotene and vitamin A in biological materials. J. Lab. Clin. Med., *28:* 904.

MCCARRISON, R. 1921 Studies in Deficiency Disease. Henry Frowde and Hodder and Stoughton, London.

MAY, C. D., AND J. F. MCCREARY 1941 The absorption of vitamin A in celiac disease. Interpretation of the vitamin A absorption test. J. Pediatrics, *18:* 200.

MAY, C. D., J. F. MCCREARY AND K. D. BLACKFAN 1942 Notes concerning the cause and treatment of celiac disease. J. Pediatrics, *21:* 289.

MITCHELL, H. H. 1943 The chemical and physiological relationship between vitamins and amino acids. Vitamins and Hormones, Academic Press, Inc., New York, *1:* 157.

MOORE, C. V., O. S. BIERBAUM, A. D. WELCH AND L. D. WRIGHT 1945 The activity of synthetic Lactobacillus casei factor ("Folic Acid") as an antipernicious anemia substance. J. Lab. Clin. Med., *30:* 1056.

MOORE, T. 1945 The interrelation of vitamins. Vitamins and Hormones, *3:* 1.

SPIES, T. D., G. C. LOPEZ, J. A. MENENDEZ, V. MINNICH AND M. B. KOCH 1946 The effect of folic acid on sprue. South. Med. J., *39:* 30.

SPIES, T. D., F. MILANES, A. MENENDEZ, M. B. KOCH AND V. MINNICH 1946 Observations on the treatment of tropical sprue with folic acid. J. Lab. Clin. Med., *31:* 227.

WILLIAMS, R. R., AND T. D. SPIES 1938 Vitamin B_1 (Thiamine) and its Use in Medicine. The Macmillan Co., New York, pp. 59–60, 68–70.

THE RELATIONSHIP OF NICOTINIC ACID, TRYPTOPHANE AND PROTEIN IN THE NUTRITION OF THE PIG [1]

R. W. LUECKE, W. N. McMILLEN, F. THORP, JR., AND CAROLYN TULL
Departments of Agricultural Chemistry, Animal Husbandry and Animal Pathology and the Institute of Nutrition, Michigan State College, East Lansing

(Received for publication October 27, 1946)

The effects of nicotinic acid deficiency in swine have been well established by Chick and co-workers ('38), Hughes ('43), Madison, Miller and Kieth ('39) and Wintrobe ('39). Recently, Wintrobe and co-workers ('45) showed that when the level of casein in a purified ration was 26%, the absence of nicotinic acid in the ration caused no apparent sign of nutritional deficiency except that of a slightly lowered growth rate. They also showed that when the level of casein in the diet was lowered to 10% and nicotinic acid excluded from the vitamin supplement, typical symptoms of nicotinic acid deficiency resulted. The relationship between protein and nicotinic acid may be explained by the work of Krehl et al. ('45, '46). These workers found that the inclusion of corn grits in a nicotinic acid-low ration caused a marked growth retarding effect when fed to rats. This effect was counteracted by supplements of either nicotinic acid or l(—) tryptophane. When either rice, oats or rye were substituted for corn, good growth was obtained which seemed to indicate that the low nicotinic acid content of corn was not the only limiting factor in its growth retarding effect. Spector and Mitchell ('46), in an application of the paired feeding technique, found that

[1] Published with the permission of the Director of the Michigan Agricultural Experiment Station as journal article no. 838 (n.s.).

the growth-depressing effect of corn on rats fed a nicotinic acid-low ration was associated with a decreased food consumption. These authors further found that the stimulation in growth produced by supplementing the corn grits ration with nicotinic acid or tryptophane was largely, but not entirely, associated with an increased caloric intake.

In view of the fact that corn constitutes a major portion of the ration of swine, particularly in the corn belt states, it seemed advisable to study the possible relationships between nicotinic acid and tryptophane in a ration largely made up of corn.

EXPERIMENTAL

Pigs used in this experiment were farrowed by Duroc Jersey sows which had been bred to a Yorkshire boar. Up to the time of weaning all animals had been kept on alfalfa pasture to prevent, as far as possible, the appearance of any known deficiency. Two weeks before the beginning of the experiment the pigs were vaccinated for hog cholera using the serum and virus method. The three basal rations used are shown in table 1. The protein content of rations A, B and C were 19.2, 14.0 and 15.9%, respectively, and the nicotinic acid contents

TABLE 1

Composition of basal ration.[1]

	RATION A	RATION B	RATION C
	%	%	%
Corn	80	87	
Oats			90
Casein (commercial)	12	5.5	4
Soybean oil meal	6	5.5	4
Complex mineral mixture[2]	2	2	2

[1] The following amounts of vitamins were supplied daily to each animal: thiamine, 10 mg; riboflavin, 10 mg; calcium pantothenate, 25 mg; and pyridoxine hydrochloride, 6 mg.

[2] This mineral mixture contained the following (in %): bonemeal, 32.3; ground limestone, 32.3; sodium chloride, 32.3; ferrous sulfate, 2.5; copper sulfate, 0.2; manganese sulfate, 0.1; zinc oxide, 0.1; cobaltous acetate, 0.1; and potassium iodide, 0.1.

were 5.22, 6.23 and 6.25 mg per pound, respectively, as determined microbiologically by the method of Krehl, Strong and Elvehjem ('43). The higher nicotinic acid content of ration B was due to the fact that a new shipment of corn was used which contained appreciably larger amounts of this vitamin. The 3 rations were supplemented with a vitamin A and D oil in amounts which supplied 1500 I.U. of vitamin A and 200 I.U. of vitamin D per pound of feed. All lots were fed ad libitum, and each pig received daily the amounts of thiamine, riboflavin, calcium pantothenate and pyridoxine hydrochloride indicated in table 1.

Both at the beginning and again during the last week of the experiment the pigs were placed in metabolism cages and a 24-hour urine collection was obtained from each animal. The daily excretion of thiamine, riboflavin, pantothenic acid, nicotinic acid and N^1-methylnicotinamide was determined. In addition the daily excretion of tryptophane was determined in lots 3, 4, 5 and 6. Thiamine in the urine was determined by the method of Mickelsen, Condiff and Keys ('45). Microbiological methods were used for the determination of riboflavin (Snell and Strong, '39), pantothenic acid (Neal and Strong, '43) and nicotinic acid (Krehl et al., '43). N^1-methylnicotinamide was determined by the method of Huff, Perlzweig and Tilden ('45). The assay for tryptophane in the urine was similar to that used by Schweigert, Säuberlich and Elvehjem ('45) who modified the nicotinic acid method of Krehl et al. ('43) by omitting tryptophane from the media and replacing it with nicotinic acid.

The animals were bled at regular intervals and a study made of the cellular constituents of the blood. At the conclusion of the experiment the animals were autopsied and a pathological study made of the affected tissues.

RESULTS

The effect of high protein

Pigs in lot 1 were fed the high protein basal ration A plus daily supplements of 30 mg of nicotinic acid. These animals

gained an average of 1.40 pounds daily as shown in table 2. This represents a higher average daily gain than was shown by the pigs in any other lot in the experiment. In addition the gains for these 5 pigs were more uniform, and all of the animals had the healthy appearance of normal pigs. The animals in this lot consumed an average of 48 mg of nicotinic acid per day. Table 3 shows that the total daily excretion of nicotinic acid plus its metabolic N^1-methylnicotinamide was 25.26 mg per pig or about 53% of the amount ingested. Autopsy of the pigs in lot 1 revealed no gross pathology.

TABLE 2

Response of pigs fed the various experimental rations.

CATEGORY OF INTEREST	LOT NUMBER					
	1	2	3	4	5	6
Ration fed	A	A	B	B	B	C
Number of pigs	5	5	5	5	5	5
Initial age in weeks	8	8	6	6	6	6
Number of weeks on trial	8	8	6	6	6	6
Average initial weight in lbs.	40	39	28	27	27	28
Average daily gain in lbs.	1.40	1.00	0.76	0.62	1.00	0.76
Average daily feed consumption, lbs.	3.64	3.09	2.27	1.98	2.29	2.29
Lbs. of feed per lb. of gain	2.61	3.09	2.98	3.19	2.29	3.01

Pigs in lot 2 were fed ration A. The animals in this lot did not appear to be as healthy as those in lot 1 and their average daily gain was smaller (table 2). Three of the animals in this lot developed a mild diarrhea within 4 weeks. The average daily excretion of N^1-methylnicotinamide was 12.21 mg per pig as compared with 22.34 mg for the animals in lot 1.

From an inspection of both the growth and excretion data the animals did not seem to be as abnormal as one would expect. The autopsy results, however, indicated an abnormal condition in the large intestine. When the abdomen was opened the large intestine appeared thickened and upon palpation felt doughy. The lesions were limited to the large intestine and particularly to that portion of the gut between the cecum and rectum. In some cases the congestion of the

mucus membrane was severe. A yellowish caseated material was firmly adherent to the mucus membrane. Small masses of feces were found adhering to the gut in the affected areas. All other organs studied appeared normal. Thus, although the protein in the ration was high the absence of nicotinic acid resulted in deficiency symptoms which were confined entirely to the large intestine. The fact that Wintrobe et al. ('45) reported no such deficiency when a nicotinic acid-low purified ration was fed suggests that corn plays an important role in the etiology of this deficiency. This further suggests that the relation between corn, nicotinic acid and tryptophane may be similar to that found for the rat (Krehl et al., '46).

The effect of low protein

Ration B containing 14% protein was fed to the pigs in lot 3. Each animal in this lot received 30 mg of nicotinic acid per day. As was expected the animals in this lot were normal in appearance although their growth rate was not as great as that of the animals in lots 1 and 2; however, some of this difference is due to the fact that the pigs in this lot were placed on the experiment at an earlier age and weighed less than the animals in lots 1 and 2 (table 2). The average daily excretion of N^1-methylnicotinamide was 15.13 mg per pig for the animals in this lot. Autopsy revealed no abnormalities of any kind, the gut being perfectly normal.

Pigs in lot 4 were fed the low protein ration B. During the fourth week of the experiment 4 of the animals in this lot developed severe diarrhea. The hair coat appeared rough and the skin around the ears was scaly. Autopsy revealed intestinal lesions similar to those described for animals in lot 2 except that they were even more severe. In some cases the colon had become so weakened that even moderate pressure would tear the walls. No pathogenic bacteria could be isolated from the intestines of these animals. The daily excretion of N^1-methylnicotinamide dropped to an average of 4.92 mg per pig. The symptoms manifested by these animals corre-

spond to those of nicotinic acid deficiency in pigs as described by other workers.

The animals in lot 4 did not develop anemia as evidenced by an average hemoglobin content of 12.55 gm per 100 ml, but a mild leucocytosis was noted. No other morphological abnormalities were observed.

Pigs in lot 5 were fed basal ration B plus daily supplements of 200 mg of d,l-tryptophane. It was evident after the first 2 weeks of the experiment that the animals in this lot were gaining weight more efficiently than pigs in the other lots. The pigs had the sleek healthy appearance of normal animals. As shown in table 2 the average daily gain was 1 lb. per pig and only 2.29 lbs. of feed were required per pound of gain. As was expected the tryptophane excretion was higher than that of lots 3 and 4, but it was surprising to find that the average daily excretion of N[1]-methylnicotinamide was 14.00 mg as compared with 4.92 mg for animals in lot 4 (table 3) since neither lot received any added nicotinic acid. Animals in lot 5 consumed 2.29 lbs. of basal ration per day which furnished 14.27 mg of nicotinic acid. The total daily excretion of nicotinic acid and N[1]-methylnicotinamide averaged 15.69 mg per pig, which is in excess of the amount ingested. It would thus appear that the animals in this lot were obtaining an additional source of nicotinic acid which is in some way related to the feeding of tryptophane. No corresponding increase in the excretion of nicotinic acid was noted. Rosen, Huff and Perlzweig ('46) have reported that when rats were fed 50 mg of either l-tryptophane or its racemic mixture, an increase in both N[1]-methylnicotinamide and nicotinic acid excretion occurred. It is possible that if larger amounts of tryptophane had been fed to the pigs the excretion of nicotinic acid would have been increased. Autopsy of the animals in this lot revealed that in 2 of the pigs a few gross lesions in the colon were observed. Although these lesions were of a minor nature and seemed to have no visible effect on the health of the pigs, it would seem that either the level of tryptophane fed was not sufficient to compensate completely for the lack of nicotinic

acid or that a source of nicotinic acid is indispensable no matter what the level of tryptophane in the diet may be.

Pigs in lot 6 were fed basal ration C made up largely of whole oats, a grain which is also low in nicotinic acid. The level of protein was 15.9% as compared with 14.0% in ration B. The growth response of these pigs was superior to that of the animals in lot 4 which had been fed the low protein corn

TABLE 3

Average daily urinary excretion of B vitamins, tryptophane and N^1-methylnicotinamide.[1]

LOT NO.	THIAMINE	RIBOFLAVIN	PANTOTHENIC ACID	NICOTINIC ACID	N^1-METHYL-NICOTINAMIDE	TRYPTOPHANE
1	1.68 [2]	2.70	14.25	2.92	22.34	
	0.40–2.50 [3]	1.05–3.42	9.63–18.00	1.92–4.51	19.10–31.81	
2	1.33	1.83	19.22	1 91	12.21	
	0.81–1.82	1.53–2.12	13.04–25.52	0.94–2.75	7.25–16.30	
3	1.86	1.72	10.19	2.28	15.13	14.25
	1.00–8.27	1.04–2.67	8.25–13.60	1.50–3.50	14.50–19.86	9.01–17.00
4	1.30	1.45	14.38	1.31	4.92	10.71
	1.02–1.65	1.11–2.16	12.43–18.06	1.07–1.57	3.46– 7.64	8.05–14.00
5	1.27	1.59	11.54	1.69	14.00	20.94
	0.78–2.00	1.04–2.63	7.80–14.56	1.08–2.31	12.32–16.03	19.00–24.21
6	5.48	1.78	17.51	1.34	8.98	19.75
	2.67–7.71	1.20–2.32	14.60–25.00	1.05–1.48	8.40– 9.61	16.01–25.10

[1] All data are in milligrams per 24 hours.

[2] Average.

[3] Range.

ration. The pigs developed a rough hair coat and were untidy in appearance but did not exhibit diarrhea. The excretion of tryptophane was almost as high as that of the animals in lot 5 which received tryptophane. The pigs in this lot ingested 13.91 mg of nicotinic acid daily and excreted 8.98 mg of N^1-methylnicotinamide. There were no intestinal lesions found at autopsy but the colons of all animals were distended with oat hulls. The poor growth rates of the pigs in this lot may be explained by the high fiber content of the ration.

Urinary excretion of thiamine, riboflavin and pantothenic acid

The daily excretion of thiamine, riboflavin and pantothenic acid was obtained from all of the experimental pigs. It is evident from table 3 that only a small proportion of the additional thiamine and riboflavin fed to the pigs was excreted in the urine. The higher average excretion of thiamine in lot 6 may be due to the fact that oats, in general, contain considerably more thiamine than does corn. It is interesting to note that in every lot the daily excretions of pantothenic acid were higher than those of the other 2 vitamins.

DISCUSSION

The results indicate that when corn constitutes a major part of the ration nicotinic acid deficiency occurs even though the percentage of protein is relatively high. Wintrobe et al. ('45) were not able to produce nicotinic acid deficiency in swine on a diet containing 26% casein, but when the casein content was lowered to 10% nicotinic acid deficiency appeared. In the present experiment it must be remembered that while the protein content of ration A was 19.2%, it contained only 12% of crude casein and for that reason undoubtedly did not contain as much tryptophane as did the high protein ration of Wintrobe et al. ('45). It is probable that had the percentage of casein in the high protein ration been increased, deficiency symptoms would not have appeared. This is borne out by the fact that the intestinal lesions of nicotinic acid deficiency produced in pigs fed the high protein ration were not as severe as those produced when the low protein ration was fed.

It has been shown by Hughes and Ittner ('42) and by Wintrobe et al. ('43) that a lack of pantothenic acid in the diet of the young pig causes marked inflammation of the intestinal tract and diarrhea. The similarity between the lesions produced in pantothenic acid deficiency and nicotinic acid deficiency have been noted. It does not seem likely, however, that pantothenic acid was a factor in the deficiency syndrome

produced in this experiment. The presence of pathogenic organisms in the intestines of the deficient pigs was eliminated as a factor since bacterological examination of sections of the inflamed colons did not reveal the presence of any of these types of organisms.

The supplementation of the low protein corn ration with tryptophane produced animals whose external appearance was normal in every respect. The animals gained an average of 1 lb. of body weight per day. They also utilized their feed more efficiently than pigs in the other lots. The fact that 2 pigs in this lot did have a mild inflammatory condition of the colon may indicate that a dietary source of nicotinic acid is still necessary even though the ration is supplemented with tryptophane. However, one might also reason that had the level of tryptophane in the ration been higher no such condition would have appeared. The fact that the inclusion of tryptophane in the ration does lead to an increase in the excretion of N^1-methylnicotinamide tends to support the latter view. The exact nature of the relationship between nicotinic acid and tryptophane is not clear. Spector and Mitchell ('46) have postulated that the increase in the excretion of nicotinic acid and N^1-methylnicotinamide following the ingestion of tryptophane indicates that tryptophane may exert a sparing action on the dietary requirement for nicotinic acid, and furthermore that the interrelationship between nicotinic acid and tryptophane may be analogous to the metabolic interrelation of choline and methionine.

The results obtained by feeding a ration largely composed of oats to young pigs seem to be overshadowed by the high fiber content of the ration. It is significant, however, that despite the low nicotinic acid content of ration C no abnormal condition was found in the intestinal tract of these pigs which probably was due to the fact that the oat protein contains considerably more tryptophane than does the protein of corn (Block, '45).

Using a purified ration the daily nicotinic acid requirement of the young pig has been estimated (Hughes, '43) to lie be-

tween 5 and 10 mg per 100 lbs. The results of the present experiment indicate that with corn as the major constituent of the ration the requirement for this vitamin may be higher.

SUMMARY

When a high protein, low nicotinic acid, corn ration was fed to weanling pigs, mild symptoms of nicotinic acid deficiency occurred. The symptoms of nicotinic acid deficiency were confined entirely to the large intestine and particularly to the colon.

The lowering of the protein content of the same corn ration to 14.0% produced very severe inflammation in the large intestine. Supplementing this ration with 200 mg of d,l-tryptophane per day per pig gave excellent growth response in the animal. Two of the animals in this lot showed a very mild inflammatory condition of the large intestine.

No symptoms of nicotinic acid deficiency were noted when the corn was replaced with oats.

ACKNOWLEDGMENTS

The authors are indebted to the Dow Chemical Company of Midland, Michigan, for their generous supply of d,l-tryptophane and to Merck and Co., Inc., Rahway, New Jersey, for their generous supply of crystalline B vitamins used in the experiment.

LITERATURE CITED

BLOCK, R. J. 1945 The amino acid composition of food proteins. Advances in Protein Chemistry, *2:* 119, Academic Press, N. Y.

CHICK, HARRIETTE, T. F. MACRAE, A. J. P. MARTIN AND C. J. MARTIN 1938 III. Curative action of nicotinic acid on pigs suffering from the effects of a diet consisting largely of maize. Biochem. J., *32:* 10.

HUFF, J. W., W. A. PERLZWEIG AND MARY W. TILDEN 1945 A simple method for the determination of N^1-methylnicotinamide in urine. Fed. Proc., *4:* 92.

HUGHES, E. H. 1943 The minimum requirement of nicotinic acid for the growing pig. J. Animal Sci., *2:* 23.

HUGHES, E. H., AND N. R. ITTNER 1942 The minimum requirement of pantothenic acid for the growing pig. J. Animal Sci., *1:* 116.

KREHL, W. A., P. S. SARMA, L. J. TEPLY AND C. A. ELVEHJEM 1946 Factors affecting the dietary niacin and tryptophane requirement of the growing rat. J. Nutrition, *31:* 85.

KREHL, W. A., F. M. STRONG AND C. A. ELVEHJEM 1943 Determination of nicotinic acid. Modifications in the microbiological method. Ind. Eng. Chem., Anal. Ed., *15:* 471.

KREHL, W. A., L. J. TEPLY, P. S. SARMA AND C. A. ELVEHJEM 1945 Growth-retarding effect of corn in nicotinic acid-low rations and its counteraction by tryptophane. Science, *101:* 489.

MADISON, L. C., R. C. MILLER AND T. B. KEITH 1939 Nicotinic acid in swine nutrition. Science, *89:* 490.

MICKELSEN, OLAF, HOWARD CONDIFF AND ANCEL KEYS 1945 The determination of thiamine in urine by means of the thiochrome technique. J. Biol. Chem., *160:* 361.

NEAL, A. L., AND F. M. STRONG 1943 Microbiological determination of pantothenic acid. Ind. Eng. Chem., Anal. Ed., *15:* 654.

ROSEN, FRED, J. W. HUFF AND W. A. PERIZWEIG 1946 The effect of tryptophane on the synthesis of nicotinic acid in the rat. J. Biol. Chem., *163:* 343.

SCHWEIGERT, B. S., H. E. SÄUBERLICH AND C. A. ELVEHJEM 1945 The free tryptophane content of human urine. Science, *102:* 275.

SNELL, E. E., AND F. M. STRONG 1939 A microbiological assay for riboflavin. Ind. Eng. Chem., Anal. Ed., *11:* 346.

SPECTOR, H., AND H. H. MITCHELL 1946 Paired feeding in the study of the counteraction by nicotinic acid and tryptophane of the growth-depressing effect of corn in rats. J. Biol. Chem., *165:* 37.

WINTROBE, M. M. 1939 Nutritive requirements of young pigs. Am. J. Physiol., *126:* 375.

WINTROBE, M. M., R. H. FOLLIS, JR., R. ALCAYAGA, M. PAULSON AND S. HUMPHREYS 1943 Pantothenic acid deficiency in swine. With particular reference to the effects on growth and the alimentary tract. Bull. Johns Hopkins Hosp., *73:* 313.

WINTROBE, M. M., H. J. STEIN, R. H. FOLLIS, JR. AND S. HUMPHREYS 1945 Nicotonic acid and the level of protein intake in the nutrition of the pig. J. Nutrition, *30:* 395.

RIBOFLAVIN DEFICIENCY IN THE DAIRY CALF

A. C. WIESE,[1] B. CONNOR JOHNSON, H. H. MITCHELL
AND W. B. NEVENS

*Division of Animal Nutrition and the Department of Dairy Husbandry,
University of Illinois, Urbana*

FOUR FIGURES

(Received for publication October 28, 1946)

Evidence (see reviews by Goss ('43) and by Najjar and Barrett ('45)) indicates that the adult ruminant does not require the B vitamins in its diet. Extensive synthesis of the B vitamins occurs in the rumen of the adult bovine; however, it has not been established as to which, if any, of the B vitamins would be required were rumen synthesis not taking place. In order to examine this question the young ruminant in which the rumen is not yet functioning was chosen for experimental study.

The synthesis of riboflavin in the rumen of both the sheep and the bovine has been adequately demonstrated by the work of McElroy and Goss ('39, '40); Wegner et al. ('40, '41) and Hunt et al. ('41, '43). In our work the requirement of the young dairy calf for riboflavin under conditions which would tend to eliminate rumen synthesis has been investigated.

EXPERIMENTAL

The experimental animals were 2 male Guernsey calves and 1 male Holstein calf. The animals, 48 hours old or less when obtained, had been receiving colostrum. They were kept in individual metal cages 5 × 6 feet in size, and equipped with

[1] Present address: Department of Agricultural Chemistry, University of Idaho.

heavy wire mesh bottoms. The experimental diet was a "synthetic milk" having the composition shown in table 1 and containing no riboflavin. The lard and wheat germ oil were homogenized with the solution of casein, cerelose and salts to produce an emulsion containing 13% solids. For complete details concerning preparation of this diet see Wiese, Johnson, Mitchell and Nevens (in press).

TABLE 1

Composition of "synthetic milk" diet.[1]

COMPONENT [1]	PER CENT	VITAMINS ADDED	MG PER KG OF LIQUID DIET
Casein, "Labco"	30.0	Thiamine	0.65
Lard	26.3	Pyridoxine	0.65
Wheat germ oil	0.3	Nicotinic acid	2.60
Salts	4.0	Calcium pantothenate	1.30
Cerelose	39.4	Inositol	26.0
		Choline	260.0
		p-Aminobenzoic acid	2.60
		Pteroyl-glutamic acid ("folic acid")	0.052
		Biotin	0.01
		2-Methyl-1, 4-naphthoquinone	0.26
		Vitamin A	5000 I.U. per day
		Vitamin D	500 I.U. per day

[1] The lard and wheat germ oil are homogenized with the solution of casein, cerelose and salts to produce an emulsion containing 13% solids. For complete details see Wiese, Johnson, Mitchell and Nevens, J. Dairy Sci. (in press).

After the calves had been on the experiment for approximately 2 weeks, the gingival surface of the cheeks and the mucosa of the tongue became hyperemic and the gums bled on applying slight pressure. Lesions were observed at the oral canthi and along the edges of the lips. These observed pathological changes are similar to the cheilosis which has been described as occurring in human riboflavin deficiency.

The condition of the calves became progressively worse. They exhibited a copious, tenacious, chalky-colored salivary secretion which was not shown by calves on other diets. In addition to an excessive and abnormal salivary secretion, ex-

cessive lachrymation was a characteristic symptom. The gains in weight were irregular because of a variable appetite. The hair appeared rough and dull and the animals shed excessively. Certain areas of the body, such as the abdomen, became practically devoid of hair. The calves scoured and the navel region became inflamed and irritated. The photographs of calf no. 14, figures 2 and 3, illustrate the appearance of dairy calves when deficient in riboflavin. The excessive lachrymation and salivation are evident in both photographs, while figure 3 shows loss of hair on the abdomen and inflammation of the navel region.

After 5 weeks on the diet, calf no. 14 was given 5 mg of riboflavin by intravenous injection. On the day following the injection the animal showed an improved appetite and decreased salivation. This improvement was only transitory as the animal again exhibited anorexia and showed excessive salivation on the second day following the injection.

Since the condition of the animal became critical, the diet was supplemented with 5 mg of riboflavin per day. The calf was kept on this supplemented diet until taken off the experiment. Within 3 days after supplementation was begun the animal's appetite improved, the hyperemia of the buccal mucosa disappeared, excessive salivation stopped, and he started to gain weight. In approximately 10 days the lesions in the corners of the mouth, along the edges of the lips and around the navel had healed. New hair began to grow in the areas from which hair had been lost. The immediate and rapid growth response in body weight can be readily seen from figure 1. The improved appearance of the animal after riboflavin administration is shown in figure 4. The lack of secretion from the eyes and mouth is evident when comparison is made with figures 2 and 3.

Calf no. 13 was given 5 mg of riboflavin per day after 6 weeks on the deficient diet. The response to riboflavin administration was the same as that of calf no. 14. The growth data for calf no. 13 are also summarized in figure 1.

Calf no. 15, which was on the deficient diet for 4 weeks only, showed the same symptoms as those reported for calves nos. 13 and 14.

During the course of the experiment periodic examinations of the eye were made with the aid on an opthalmoscope. Neither vascularization of the cornea nor opacity of the lens was observed.

At weekly intervals 24-hour urine collections were made and the riboflavin content determined by the method of Najjar ('41). The data are given in table 2. The first collection was

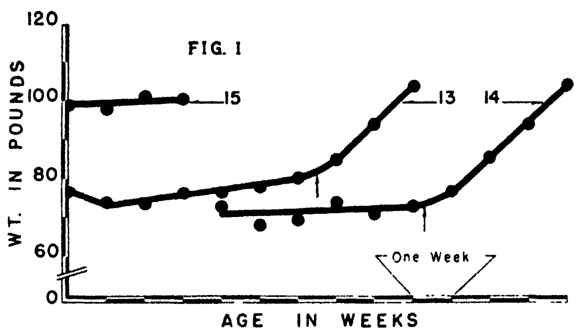


Fig. 1 Growth of calves on riboflavin deficient diet. Calves nos. 13 and 14, Guernseys; calf no. 15, Holstein. The arrow indicates when feeding of 5 mg of riboflavin per day in diet began.

made when the animals were 48 hours old and had received colostrum. Under these conditions the amount of riboflavin excreted was high. After 1 week on the riboflavin-deficient diet there was a marked drop in the amount of riboflavin excreted and by the third week little or no riboflavin was found in the urine. When riboflavin was added to the diet, however, there was an immediate rise in the urinary riboflavin excretion. The practical disappearance of riboflavin from the urine of the calves on a diet devoid of this vitamin suggests strongly that synthesis of the vitamin was not occurring, either in the intestinal tract or in the tissues. These results are in contrast to those secured with nicotinic acid, a vitamin not necessary in dairy calf nutrition. On a ration free of this

Figs. 2 and 3 Calf no. 14 after being on riboflavin deficient diet for 5 weeks. Note excessive salivation and lachrymation. Figure 3 shows loss of hair on abdomen and inflammation around navel.

Fig. 4 Calf no. 14 after receiving riboflavin supplement for 3 weeks. Note absence of symptoms.

vitamin, the urinary excretion is quite constant and of considerable magnitude (Johnson et al., in press).

When the animals were sacrificed at the close of the experiment, postmortem examination failed to reveal any internal gross lesions. Sections from the brachial and sciatic nerves, stained with hematoxylin-eosin and Mahon's technique, did

TABLE 2

The urinary excretion of riboflavin.

WEEKS ON DIET	CALF 13		CALF 14		CALF 15	
	Riboflavin fed	Riboflavin excreted	Riboflavin fed	Riboflavin excreted	Riboflavin fed	Riboflavin excreted
	mg/day	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*
0	0	7.59	0	10.59	0	1.16
1	0	0.08	0	0.09	0	0.02
2	0	0.040	0	0.01	0	0.07
3	0		0	0.06	0	
4	0	0.06	0	0.08	0	0.06
5	0	0.0	5	0.78	0	0.17
6	0	0.0	5	1.38		
7	5		5			
8	5	0.38	5	1.44		

not show any microscopic lesions. The fresh weights of the rumen-reticulum for calves 13, 14 and 15 were, respectively, 381, 356 and 349 gm. as compared with weights of 376, 407 and 360 gm for the omasum-abomasum. These measurements reveal the undeveloped condition of the rumen.

DISCUSSION

The young dairy calf fed a "synthetic milk" diet requires riboflavin. The low amounts of riboflavin excreted in the urine on a diet devoid of this vitamin indicate that appreciable synthesis of the riboflavin in the rumen or in the tissue did not occur. Furthermore, the weight of the rumen and reticulum compared to the weight of the omasum and abomasum suggest that the development of the rumen was small. The fact that after feeding riboflavin, the animal gained weight, improved in physical appearance and excreted in the urine

only a portion of the riboflavin administered is further evidence that this factor is needed by the calf.

The absence of lesions in the sciatic or brachial nerves, and possibly in the eye, may be due to the short period of time that the animals were on the deficient diet.

SUMMARY

1. The bovine species requires riboflavin, supplied either in the diet or by rumen or intestinal synthesis. On a riboflavin deficient diet the calf develops definite pathological symptoms and riboflavin practically disappears from the urine.

2. Riboflavin deficiency in the young dairy calf is characterized by hyperemia of the buccal mucosa, lesions in the corner of the mouth, along the edges of the lips and around the navel, loss of appetite, poor growth, scours, excessive salivation and lachrymation, and loss of hair. No vascularization of the cornea or opacity of the lens were observed on periodical examination with the ophthalmoscope.

3. The administration of riboflavin rapidly cures the deficiency.

LITERATURE CITED

Goss, H. 1943 Some peculiarities of ruminant nutrition. Nutrition Abs. and Rev., *12:* 531.

Hunt, C. H., E. W. Burroughs, R. M. Bethke, A. F. Schalk and P Gerlaugh 1943 Further studies on riboflavin and thiamine in the rumen content of cattle. II. J. Nutrition, *25:* 207.

Hunt, C. H., C. H. Kick, E. W. Burroughs, R. M. Bethke, A. F. Schalk and P. Gerlaugh 1941 Studies on riboflavin and thiamine in the rumen content of cattle. J. Nutrition, *21:* 85.

Johnson, B. C., A. C. Wiese, H. H. Mitchell and W. B. Nevens The metabolism of nicotinic acid and its role in the nutrition of the calf. J. Biol. Chem., in press.

McElroy, L. W., and H. Goss 1939 Report on four members of the vitamin B complex synthesized in the rumen of the sheep. J. Biol. Chem., *130:* 437.

—— —— 1940 A quantitative study of vitamins in the rumen contents of sheep and cows fed vitamin-low diets. I. Riboflavin and vitamin K. J. Nutrition, *20:* 527.

Najjar, V. A. 1941 The fluorometric determination of riboflavin in urine and other biological fluids. J. Biol. Chem., *141:* 355.

NAJJAR, V. A., AND R. BARRETT 1945 The synthesis of B vitamins by intestinal bacteria. Vitamins and Hormones, *3:* 23.

WEGNER, M. I., A. N. BOOTH, C. A. ELVEHJEM AND E. B. HART 1940 Rumen synthesis of the vitamin B complex. Proc. Soc. Exp. Biol. Med., *45:* 769.

———— 1941 Rumen synthesis of the vitamin B complex on natural rations. Proc. Soc. Exp. Biol. Med., *47:* 90.

WIESE, A. C., B. C. JOHNSON, H. H. MITCHELL AND W. B. NEVENS Synthetic rations for the dairy calf. J. Dairy Sci., in press.

ACKNOWLEDGMENTS

We wish to acknowledge the assistance of Dr. L. E. Boley and Dr. H. Hardenbrook of the College of Veterinary Medicine for the postmortem examinations; of Dr. P. H. Tracy and Mr. V. L. Swearingen of Dairy Manufactures for homogenization of the "synthetic milk"; of Mr. N. F. Mosser of the Department of Dairy Husbandry for securing the calves used in the experiment; of Dr. G. LeRoy Porter of the Carle Hospital Clinic, Urbana, for the eye examination, and of Mrs. Shirley Spaeth for the riboflavin analyses.

This investigation was supported by funds donated by Swift and Co., Chicago, Ill., and carried out with the advice of a committee appointed by the Director of the Agricultural Experiment Station consisting of the following members: H. E. Carter, T. S. Hamilton, B. C. Johnson, W. B. Nevens, H. E. Robinson, H. Spector, A. C. Wiese and H. H. Mitchell, Chairman.

We are also indebted to Hoffmann-LaRoche, Inc., Nutley, N. J. through the courtesy of Dr. J. C. Bauernfeind, for supplies of thiamine, riboflavin, nicotinic acid, pyridoxine, calcium pantothenate and biotin; to Merck and Co., Rahway, N. J., for choline; to Corn Products Refining Co., New York, N. Y. for inositol; to Lederle Laboratories, Inc., Pearl River, N. Y., through the courtesy of Dr. E. R. L. Stokstad, for pteroyl-glutamic acid and to Gelatin Products, Detroit, Mich., for vitamin A and D capsules.

THE FORMATION OF CAPILLARIES AND OTHER TISSUE CHANGES IN THE CORNEA OF THE METHIONINE-DEFICIENT RAT

JOSEPH L. BERG,[1] EDGAR R. PUND, V. P. SYDENSTRICKER, W. KNOWLTON HALL, LESTER L. BOWLES AND CHARLES W. HOCK

Departments of Pathology, Medicine, Biochemistry and Microanatomy, University of Georgia School of Medicine, Augusta

TWELVE FIGURES

(Received for publication October 12, 1946)

Corneal vascularization is a striking development which may appear with nutritional deficiencies. Hall, Sydenstricker, Hock and Bowles ('46) have described the appearance of corneal vascularization in rats fed a diet lacking protein. A similar vascularization may result from deficiencies of lysine or tryptophane (Totter and Day, '42). In a comprehensive investigation of the changes in morphology resulting from amino acid deficiencies Maun, Cahill and Davis ('45a, '45b) observed that while a diet deficient in phenylalanine resulted in no significant ocular changes, leucine deficiency caused pronounced lesions of the anterior portion of the eye. The corneal epithelium was thinner and there was a loss of cellular polarity. The epithelial cells appeared to be in flattened layers with keratinization on the surface. The basal cells stained more deeply with hematoxylin and showed increased numbers of mitoses. The substantia propria appeared to have lost its fibrillar structure and become homogenous and was thickened. Prominent dilated blood vessels were observed beneath the

[1] Present address, Department of Ophthalmology, Washington University, St. Louis 10, Mo.

epithelium and in the superficial portion of the substantia propria. Around these and in the adjacent stroma small collections of granulocytes were present. Descemet's membrane was slightly thickened. The vascularization and the epithelial metaplasia were observed to be more evident near the limbus. The same changes were observed in rats on a histidine-deficient diet although the changes were less striking (Maun, Cahill and Davis, '46). The structure of the substantia propria was preserved though it also appeared thickened. The leukocytic infiltration of the perivascular tissue was considerably less.

Glynn, Himsworth and Neuberger ('45) investigated the pathology resulting from deficiency of the sulfur-containing amino acids but made no report concerning the eyes of their animals.

That methionine deficiency in the rat may result in corneal vascularization has already been reported by Sydenstricker, Hall, Hock and Pund ('46). The present study is concerned with a more comprehensive investigation of the corneal changes which occur in this deficiency.

EXPERIMENTAL

Rats of a Wistar strain were placed on the experimental diets when from 21 to 32 days of age. The composition of these diets is given in table 1. Since Hall and Sydenstricker ('47) have pointed out that 9% casein diets such as diets 25 and 36 are suboptimal with respect to histidine, lysine, valine, threonine and tryptophane, groups of rats were placed on diets to which adequate amounts of these amino acids had been added (diets 38 and 39). The animals which received the diet containing 9% of casein (diet 25) were also compared with groups which received diets containing 10 and 11% casein (diets 22 and 23) and the control animals (those receiving diets 36 and 38) were compared with a group which received a 20% casein diet (diet 31). Where possible, littermates were divided between the various diets.

As a further control, dl methionine was fed to 3 rats having corneal vascularization in order to induce a regression of the corneal vascularization.

Also included in table 1 are figures showing the incidence of corneal vascularization in the groups of rats in the various diets as well as the changes in weight which occurred during the first 7 weeks on the diet. It is to be noted, however, that in

TABLE 1

Composition of diets, with the associated incidence of corneal vascularization and rates of growth.[1]

DIET NO	25	22	23	36	38	39	31
				Gm per 100 gm of diet			
Casein [2]	9.0	10.0	11.0	9.0	9.0	9.0	20.0
dl Methionine				0.5	0.4		
Amino acid mixture [3]					1.57	1.57	
Sucrose	81.8	80.8	79.8	81.3	79.83	80.23	70.8
No. of rats on diet	42	6	10	10	8	9	10
No. showing corneal vascularization	21	5	2	0	0	8	0
Growth, gm per day	0.5	0.2	0.0	0.9	3.1	0.7	3.0

[1] All the above diets contained in each 100 gm: salt mixture 4 gm, cod liver oil 2 gm, cottonseed oil 3 gm, choline chloride 0.2 gm, calcium pantothenate 2.0 mg, pyridoxine hydrochloride 0.4 mg, thiamine hydrochloride 0.4 mg, riboflavin 1.6 mg. The salt mixture was the same as that used by McKibben, Madden, Black and Elvehjem ('39).

[2] Vitamin test casein, General Biochemicals, Inc.

[3] Amino acids added were 0.25% of l (–) histidine hydrochloride, 0.52% of l (–) lysine hydrochloride, 0.30% of dl valine, 0.40% of dl threonine and 0.40% of dl tryptophane.

the groups of rats on the deficient diets, many died during this period. A description of the methods used in caring for the animals, techniques used in biomicroscopic examination of the rats' eyes, and of the method used in making injected corneal preparations is given in an earlier paper (Bowles, Allen, Sydenstricker, Hock and Hall, '46).

For morphologic studies, eyes showing changes typical of methionine deficiency were selected with the use of the bio-

microscope. After the animals were killed, the eyes were fixed in 1:10 formalin for 24 hours, following which the lenses were removed through incisions in the posterior portion of the eyes to facilitate sectioning. No histological changes in the lens had been observed to occur as a result of the methionine deficiency. The tissue next was placed successively in 80%, 95% and absolute ethyl alcohol for periods of 1 hour each, after which it was cleared in Cedar oil for a minimum of 48 hours, or until such time as the procedure was to be completed. After immersion in paraffin for 12 hours at 55°C., the tissues were blocked and serial sections 7 microns thick were cut from a representative area of the cornea. The sections were stained with the Harris hematoxylin stain followed by an eosin counterstain.

Flat preparations also were made from quadrants of corneas of methionine-deficient rats using the method of Buschke, Friedenwald and Fleischmann ('43). One such preparation was made from a cornea the capillaries of which had been injected with India ink in the manner described by Bowles, Allen, Sydenstricker, Hock and Hall ('46). All flat preparations were stained with Harris hematoxylin stain followed by an eosin counterstain.

The histological studies reported here were all made on corneas from methionine-deficient rats which had been fed diet 39, although no differences were observed between the histological changes resulting from consumption of this diet and the other methionine-deficient diets. Four of the rats had been on the diet for about 4 months. The other 5 on this diet died after intervals varying from 5 weeks to 4 months.

The normal variations in the histology of the cornea in this strain of rats had been determined by the study of a number of normal-appearing corneas from control and stock rats.

As in previous studies our use of the term "corneal vascularization" is confined to the situation where there is an actual invasion of the cornea by capillaries. In another paper we have described the normal variation in vascularity

at the limbus in the rat (Bowles, Allen, Sydenstricker, Hock and Hall, '46).

RESULTS AND DISCUSSION

Gross examination of the corneas of the methionine-deficient rats revealed no observable changes other than a transient, diffuse haziness of the cornea. This corresponded to a moderate degree of opacity which was observed with the biomicroscope preceding the invasion of the cornea by capillaries. In general, the capillary invasion and the regression of the corneal vascularization when the rats were changed to the control diet, as observed with the biomicroscope, were similar to what had been described as occurring in protein deprivation (Hall, Sydenstricker, Hock and Bowles, '46). The principal deviation was that the vessels were usually much smaller and often less numerous than the corneal vessels observed in the protein-deficient rats although eventually, in some of the rats, the vessels attained a comparable size. In many rats which had been on the methionine-deficient diet for a month or more, the vessels were very small, and barely visible with the biomicroscope. Frequently they were only partially filled or bloodless. This might be due to a reduction in blood volume similar to that observed by Metcoff, Favour and Stare ('45) and by Benditt, Straube and Humphrey ('46) in rats fed diets very low in protein, or it might possibly be due to spontaneous regression of the vascularization. Glynn, Himsworth and Neuberger ('45) observed hypoproteinemia in their rats deficient in the sulfur-containing amino acids which it is presumed might result in a reduced blood volume. Except for the haziness of the cornea just preceding the formation of the capillaries, the corneas usually appeared to be clear and transparent.

Photophobia was commonly observed in the experimental animals showing corneal changes, as has been noted in other nutritional deficiencies affecting the cornea.

Figures 1 through 4 are pictures of injected rat corneas with different degrees of vascularization. The size of the

vessels seen in figure 5 is characteristic of a large proportion of the corneal vascularization seen in biomicroscopic examination of methionine-deficient rats. The general pattern of vascularization in the corneas of the methionine-deficient rats seemed to be very similar to that observed to result from protein deprivation (Hall, Sydenstricker, Hock and Bowles, '46) though the capillaries were usually much smaller. The peculiar pattern of vascularization shown in the cornea in figure 6 is exceptional. Since this pattern of vascularization was observed in only one eye of one of the methionine-deficient rats, it is quite possible that it may have been due to trauma of some sort, although no evidence of trauma was seen during weekly biomicroscopic examinations or in the injected preparation. Figure 4 is a picture of the cornea of the other eye of this rat.

It will be noted in examining the pictures of the injected corneas that a number of vessels may be seen which are unfilled or are poorly filled with the injection fluid. In most cases these vessels, as well as some which did fill with the injection fluid, were those frequently observed to be bloodless on biomicroscopic examination when the deficiency had reached an advanced stage. The distal ends of many of the capillaries seen in the pictures were probably in many instances, at least for a short distance, newly formed capillaries, as yet nonfunctional. Figure 3 shows not only vessels which were not filled with the injection fluid when the injection was made, but also shows the beading such as was observed during the process of regression of vascularization in protein-deficient rats when these rats were returned to an adequate diet (Hall, Sydenstricker, Hock and Bowles, '46). This rat had been continuously on the deficient diet from the beginning of the experiment until the time when the injection preparation was made. It is possible that this represents a partial spontaneous regression of the vascularization. The 9% casein diets which we used contained 0.22 to 0.30% of methionine as compared to the rat's requirement of 0.6% in the diet (Hall and Sydenstricker, '47).

The plexus of very fine vessels such as may be seen in the middle of the picture in figure 4 is such as is frequently observed in corneal vascularization resulting from amino acid deficiencies.

On examining the histological sections of the cornea, the most constant finding was edema of the epithelium and substantia propria. In the epithelium the edema was both intracellular (fig. 7) and intercellular (fig. 8).

Karyorrhexis, or swelling of the cells with granular disintegration of the nuclear chromatin, was present and almost always in the outer 3 cell layers of the corneal epithelium.

Bowman's membrane was rarely affected and when it was, it was usually slightly displaced in a wavy fashion (fig. 7).

Vascularization was most frequently characterized by subepithelial capillaries (fig. 9) and the more marked the vascularity, the deeper the vessels were found in the stroma. Usually the vessels were seen at approximately the position observed with the biomicroscope. In flat preparations uncanalized vessels were seen and these undoubtedly were the same structures which, when seen with the biomicroscope, were described as fine, white lines (fig. 11).

The vascularization in the cornea which we observed in methionine deficiency was similar to that described by Maun, Cahill and Davis ('46a, '46b) for leucine and histidine deficiencies, though the detailed cellular changes do not coincide. However, it must be pointed out that our study was made with rats which had been for a much longer period on a diet less drastically deficient than those used by Maun, Cahill and Davis.

After methionine-deficient rats had been returned to the control diet and vessels were no longer visible on biomicroscopic examination, small bloodless vessels still could be found in histological sections.

The term "corneal corpuscle" is generally used to refer to the normal cell elements of the substantia propria, of which there are 2 forms: (1) fixed supporting cells, and (2) wandering phagocytes. Our observations indicate that under certain

conditions a third form may arise, this form being by function a primitive endothelial cell, as is indicated by the following observations. On examination of the area of formation of the new capillary, increased numbers of cells were always observed distal to the end of the new capillary. These cells were similar to the normal corneal corpuscle of the phagocytic type, but varied somewhat in size and shape. There were present all gradations from the usual corneal corpuscle to a cell type which was larger and longer. This latter type appeared identical with the endothelial cells seen in the capillary walls. As seen in serial sections, these cells were irregularly arranged and were seen at various depths. However, in flat corneal preparations, the integration of these cells could be followed from an irregular arrangement to their final organization into a functional capillary. This integration may be seen in figures 10, 11 and 12. Figure 10 is from a photomicrograph of a flat preparation of a cornea, the capillaries of which had been injected with India ink. The area shown in the picture includes the branched end of a capillary at the farthest point reached by the injection fluid. As may be observed in this picture, when the side walls of the capillary are in focus, they may be seen as orderly lines of cells. On following the capillary towards the distal end, a point is reached where it becomes bloodless and the lines of cells forming the walls are less orderly in arrangement, as may be seen in figure 11. The 2 lines of cells forming the walls may diverge slightly, and from this point the cells are spaced farther apart and become progressively more irregular in arrangement, until only scattered cells may be observed, as in figure 12. Study of the section from which figure 12 was made showed all gradations in size and shape from typical phagocytic type corneal corpuscles to the formed endothelial cells, as previously described.

That endothelial cells may be derived from corneal corpuscles may be supported on an embryological basis. It is generally accepted that in the embryo amoeboid mesenchymal

cells move in between the optic cup and the epidermis to form, through differentiation, the substantia propria, which further differentiates to form Bowman's and Descemet's membranes. The corneal corpuscles of the substantia propria are apparently these primitive mesenchymal cells, some of which are differentiated to form the supporting cells, and some of which are histiocytes which retain their primitive amoeboid nature. These histiocytes may function as phagocytes, or may further differentiate into endothelial cells when capillaries are needed. In this instance, it is suggested that with methionine deficiency, the nourishment of the avascular corneal tissue becomes inadequate and an attempt is made by the tissue to compensate for the inadequacy by the development of capillaries to bring additional nourishment.

Of the 9 rats from 4 litters on diet 39, 1 died before corneal vascularization appeared. The remaining 8 all developed definite and extensive corneal vascularization in from 6 to 102 days (mean, 35 days). This diet (diet 39), where the nutritional deficiency was more specific, resulted in a rather prompt production of extensive corneal vascularization in 8 out of 9 rats while the other methionine-deficient diets, where the deficiency was less specific, resulted in corneal vascularization in only about half of the rats. The corneal changes resulting with these diets were frequently less extreme and more variable in time of appearance. However, there were no qualitative differences in the corneal changes produced in the various methionine-deficient diets. It is of interest that complete starvation does not produce corneal vascularization (Bessey and Wolbach, '39) although elimination of all protein from an otherwise adequate diet results in prompt and extensive corneal vascularization (Hall, Sydenstricker, Hock and Bowles, '46).

As in rats deprived of protein (Sydenstricker, Hall, Hock and Pund, '46), the repeated administration of large doses of riboflavin had no observable effect on the corneal vascularization resulting from methionine deficiency.

The growth of the rats on the various methionine-deficient diets used seemed to depend more on the age at which the rats were started rather than on the identity of the diet.

None of the control rats showed any significant corneal changes.

SUMMARY

The development of corneal vascularization in rats fed a diet deficient in the sulfur-containing amino acids was followed with the biomicroscope. Histologic studies confirmed these observations. Vascularization was noted first in the subepithelial tissue, and when pronounced, vessels appeared in the deeper portions. Non-canalized capillaries were observed. A difference in the cellular components of the substantia propria was noted and the significance of the endothelial-like cells as to their relation with newly formed capillaries is discussed.

Degenerative changes of the epithelial cells of the cornea are described.

No histologic changes in the lenses were seen.

ACKNOWLEDGMENT

This study was aided by grants from The John and Mary R. Markle Foundation and from Merck and Co. We wish to thank Dr. Harris Isbell, Dr. Sam Singal and Dr. Lane Allen for suggestions and assistance. Christina Yates and Elizabeth J. Thompson cared for the animals. Josephine Hatch prepared the histological sections.

LITERATURE CITED

Benditt, E. P., R. L. Straube and E. M. Humphreys 1946 The determination of total circulating serum proteins and erythrocyte volumes in normal and protein depleted rats. Proc. Soc. Exp. Biol. Med., *62:* 189.

Bessey, O. A., and S. B. Wolbach 1939 Vascularization of the cornea of the rat in riboflavin deficiency with a note on corneal vascularization in vitamin A deficiency. J. Exp. Med., *69:* 1.

Bowles, L. L., L. Allen, V. P. Sydenstricker, C. W. Hock and W. K. Hall 1946 The development and demonstration of corneal vascularization in rats deficient in vitamin A and in riboflavin. J. Nutrition, *32:* 19.

BUSCHKE, W., J. S. FRIEDENWALD AND W. FLEISCHMANN 1943 Studies on the mitotic activity of the corneal epithelium. Methods. The effects of colchicine, ether, cocaine and ephedrin. Bull. Johns Hopkins Hosp., *73:* 143.

GLYNN, L. E., H. P. HIMSWORTH AND A. NEUBERGER 1945 Pathological states due to deficiency of the sulfur-containing amino acids. Brit. J. Exp. Path., *26:* 326.

HALL, W. K., AND V. P. SYDENSTRICKER 1947 The .production of methionine deficiency in the rat with low casein diets. Arch. Biochem., *12:* 147.

HALL, W. K., V. P. SYDENSTRICKER, C. W. HOCK AND L. L. BOWLES 1946 Protein deprivation as a cause of corneal vascularization in the rat. J. Nutrition, *32:* 509.

MCKIBBEN, J. M., R. J. MADDEN, S. BLACK AND C. A. ELVEHJEM 1939 The importance of vitamin B_6 and factor W in the nutrition of dogs. Am. J. Physiol., *128:* 102.

MAUN, M. E., W. M. CAHILL AND R. M. DAVIS 1945a Morphologic studies of rats deprived of essential amino acids: I. Phenylalanine. Arch. Path., *39:* 294.

——— 1945b Morphologic studies of rats deprived of essential amino acids. II. Leucine. Arch. Path., *40:* 173.

——— 1946 Morphologic studies of rats deprived of essential amino acids: III. Histidine. Arch. Path., *41:* 25.

METCOFF, J., C. B. FAVOUR AND F. J. STARE 1945 Plasma protein and hemoglobin in the protein deficient rat. A 3-dimensional study. J. Clin. Invest., *24:* 82.

SYDENSTRICKER, V. P., W. K. HALL, C. W. HOCK AND E. R. PUND 1946 Amino acid and protein deficiencies as causes of corneal vascularization: A preliminary report. Science, *103:* 194.

TOTTER, J. R., AND P. L. DAY 1942 Cataract and other ocular changes resulting from tryptophane deficiency. J. Nutrition, *24:* 159.

PLATE 1

EXPLANATION OF FIGURES

(All 15 × reduced approximately one-third)

1 through 5 Oblique views of corneas of methionine-deficient rats, which have been injected with India ink. These rats had been on a methionine-deficient diet for 116, 116, 200, 40 and 160 days, respectively, when the injection preparation was made, although corneal vascularization had been observed with the biomicroscope in these rats after 39, 99, 180, 29 and 98 days on the deficient diets.

6 Anterior view of the injected cornea from the other eye of the rat, the cornea from which is shown in figure 4.

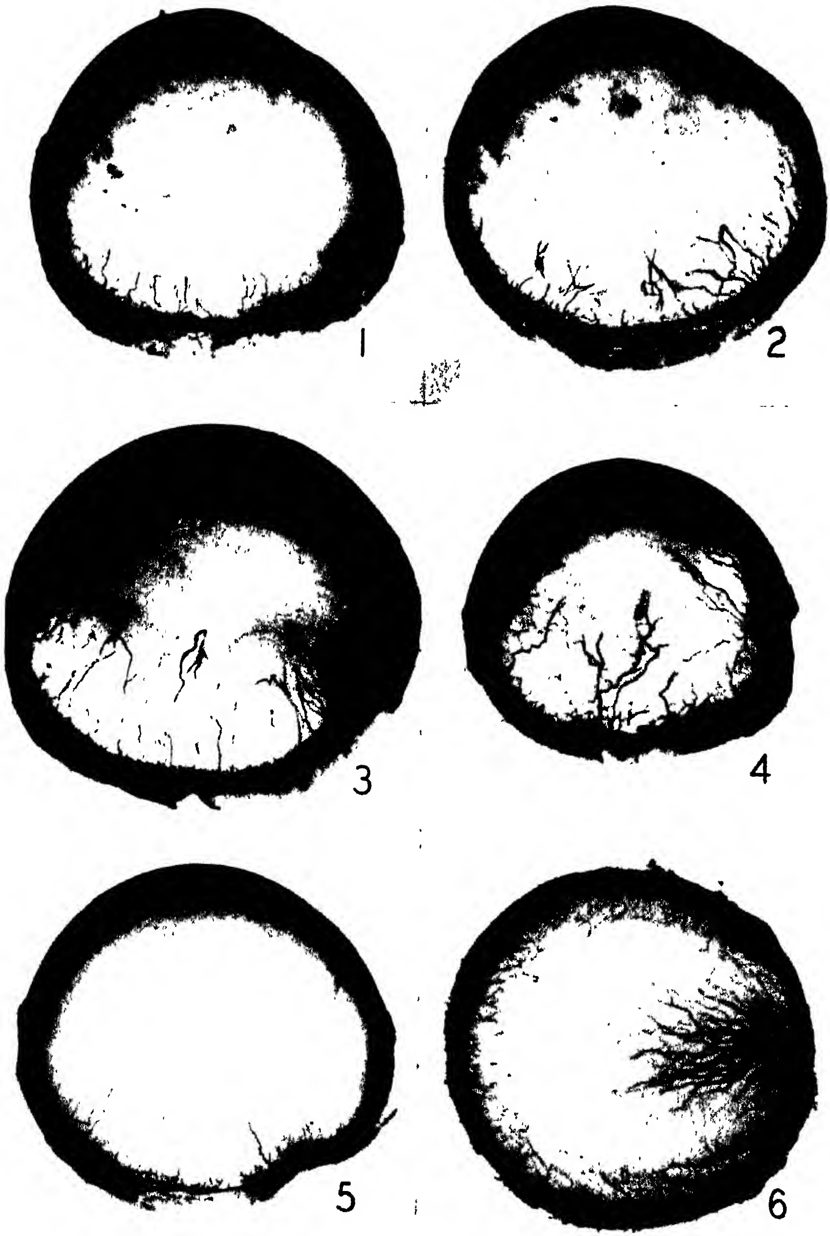

1
2
3
4
5
6

PLATE 2

EXPLANATION OF FIGURES

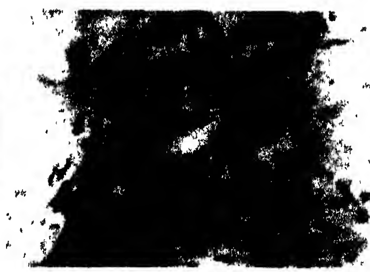
7 through 9 Sections of corneas of methionine-deficient rats. Figure 7 shows intracellular edema of the epithelial cells and wavy displacement of Bowman's membrane. Figure 8 shows early intercellular edema of the epithelial cells. Figure 9 shows focal areas of karyorrhexis of the nuclear chromatin of the outer layers of cells, pyknosis of the nuclei of the inner layers of cells, and a large subepithelial capillary. (Figure 7, 600 ×; figs. 8 and 9, 1400 ×. All reduced approximately one-third).

10, 11 and 12 Flat preparations of corneas showing capillary formation. (Figure 10, 430 ×; figs. 11 and 12, 700 ×. All reduced approximately one-third).

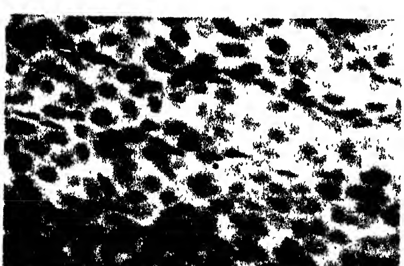
7

8

9



10



11

12

WORK PERFORMANCE, RESPIRATORY EXCHANGE, AND CERTAIN BLOOD CONSTITUENTS AFTER ISOCALORIC MEALS OF LOW AND HIGH CARBOHYDRATE CONTENT [1,2]

JOHN HALDI AND WINFREY WYNN

Department of Physiology, Emory University, Emory University, Georgia

ONE FIGURE

(Received for publication October 19, 1946)

In previous studies (Haldi and Wynn, '46a, b) it was found that work performance in severe exercise of short duration was not affected by either the amount or kind of food ingested several hours before exercise. Hypoglycemia with a concomitant diminution in the capacity for work, believed by some to be the resultant of a high carbohydrate intake (Wilder, '43; Thorne, Quinby and Clinton, '43) was not observed in any of our subjects after a meal rich in carbohydrate. The limiting factor of endurance for brief exhausting work, however, may not be the same as for heavy work that can be carried on for a longer time before exhaustion. This consideration suggested the advisability of the present study on work performance after different types of meals with the work load so adjusted that exercise could be continued for a much longer time than in our previous experiments.

The subjects were 3 adult males, 2 adolescent males, and 1 adult female, ranging in age from 14 to 37 years and in weight from 120 to 200 pounds. This purposeful diversification in the

[1] Preliminary report: Fed. Proc., '46; *5:* 39.

[2] Acknowledgment is made to the Sugar Research Foundation for a grant-in-aid for conducting this investigation.

choice of subjects was designed to determine whether there were any differences in response to the 2 types of meals that might be related to age or sex. All the subjects were in splendid physical condition and accustomed to 1 form or another of athletic activity.

PROCEDURE

For a period of about 3 weeks each subject was thoroughly trained in riding the Prony-brake bicycle ergometer against a load of such magnitude that he was able to continue the exercise from 12 to 18 minutes before becoming completely exhausted. This load, which had been determined for each subject by a number of trials, was maintained constant throughout the experiments. In the training program the subjects while riding the bicycle were connected with the metabolism apparatus through the mouthpiece and nose clip. The apparatus used in these experiments has been described elsewhere (Bachmann and Haldi, '37). At the conclusion of the training period the amount of work done was fairly constant from day to day. Blank experiments were then run to familiarize the subject with the entire procedure.

In the earliest stages of this study we were concerned over establishing valid criteria of complete exhaustion. It was soon found, however, that with the whole-hearted cooperation given by the subjects this matter presented no serious difficulties. Each subject continued riding until he reached a point where it was impossible with maximum effort to push down the pedal on the bicycle. The speed of riding as shown by a speedometer remained constant throughout the experiment. As the rider neared exhaustion there was observed a profuse perspiration over the entire body and twitching of the muscles. On a number of occasions the subjects experienced severe cramps in the calves of the legs.

On the day of the experiment the meal prepared in the University cafeteria by competent dietitians was served at noon. Each meal supplied approximately 1050 cal. Carbohydrate, protein, and fat provided, respectively, 76, 13, and 11%

of the total calories in the high carbohydrate meal and 13, 37, and 50%, respectively, in the low carbohydrate meal. All the experiments on 1 subject were completed before beginning with another. Two experiments were done each week, 1 on each of 2 different types of meals, and were so spaced as to leave several days intervening. On these intervening days the subject rode the bicycle ergometer against the customary load for 10 minutes in order to keep himself in training. There were 6 experiments on each subject with each kind of meal, making a total of 72 experiments.

One and a half to 2 hours after the meal the subject reported to the laboratory and reclined on the metabolism couch for 30 minutes. He was then connected to the metabolism apparatus and the respiratory exchange determined for 2 10-minute periods. At the conclusion of these base-line periods blood samples were drawn by finger puncture for sugar determinations on all the subjects and also for lactic acid analyses on 4 of them. The subject then mounted the bicycle ergometer, adjusted the mouthpiece so as to expire into the spirometer and at a given signal started pedalling at the rate of 56 revolutions per minute. He continued at this rate until exhausted, whereupon he rolled off the bicycle onto the adjacent couch without being disconnected from the metabolism circuit. Readings on the counter of the ergometer were taken after 10 minutes riding and again at the conclusion of the exercise. The subject rested for 10 minutes in the recumbent position and then mounted the bicycle for the second time and rode again until exhausted. At the end of this second exercise period he reclined on the couch while determination of the respiratory exchange was continued for 3 10-minute recovery periods. Blood samples were drawn immediately after the 2 exercise periods and again at the end of 30 minutes of recovery. Analysis for blood sugar was made by the Hagedorn-Jensen procedure (Peters and Van Slyke, '32) and for lactic acid by the Barker-Summerson method ('41).

RESULTS

Work output

The amount of work done varied from 1 subject to another but within a relatively narrow range for 4 of them: W.W., C.D., J. L., and J.S. The 1 female subject accomplished only about 65% as much work as these 4 whereas 1 adolescent male did 20% more work than the other 4 males. These latter results are of interest for here we have a youth 14 years old with a much greater capacity for work than was manifested

TABLE 1

Work output and per cent recovery.[1]

Subject	High carbohydrate meal: Work done, First period	High carbohydrate meal: Work done, Second period	High carbohydrate meal: Recovery	Low carbohydrate meal: Work done, First period	Low carbohydrate meal: Work done, Second period	Low carbohydrate meal: Recovery
	kg m	*kg m*	%	*kg m*	*kg m*	%
W.W.	13,993	6,933	49.5	13,456	6,856	51.0
J.L.	14,435	8,076	56.0	13,666	6,713	48.9
C.D.	13,643	7,096	52.0	13,324	7,802	58.6
J.H.	16,819	9,590	56.9	16,374	9,311	56.9
J.S.	13,830	7,054	50.9	13,397	6,583	48.9
E.H.	9,128	4,892	53.6	9,082	5,334	57.6
Average	13,641	7,273	53.2	13,216	7,100	53.7

[1] Each figure in the table is an average of 6 experiments except the final averages which are derived from 36 experiments.

by a robust adult (J.L.) who had engaged in professional football only a short time previous. The other adolescent (J.S.) who weighed only 130 lbs. and the adult (C.D.) weighing 120 lbs. were able to do almost as much work as the heavier football player. Obviously muscle mass was not the sole factor determining work output.

Inspection of the data in table 1 shows a uniformity of results with all the subjects on the 2 types of meals. The average amount of work done in the first exercise period and in the first and second periods combined was approximately 3% more after the high than after the low carbohydrate meal.

This difference, however, was not statistically significant. There was likewise no significant difference in the percentage recovery after the 2 meals. The percentage recovery was determined by multiplying the work done in the second period by 100 and dividing the product by the work done in the first period.

The double work period was the method of choice not only because it provides more information than the single work period, but also because it is held by some investigators (Foltz, Ivy and Barborka, '42) that the percentage recovery is less variable in untrained individuals and consequently more advantageous for the study of fatigue than a single work period. With our subjects, however, it was found that the average of the coefficients of variation for the work done during the first exercise period was 6.0 as compared with a coefficient of 13.7 for the percentage recovery. By this criterion the work done in the single period was 44% as variable as the percentage recovery.

The differences in our results as compared with those of Foltz and others ('42) were not due to an unusually high coefficient in the percentage recovery which was 13.7 in our subjects as compared with their coefficient of 11.4 but to our low coefficient of 6.0 in the work output of the first period as against their coefficient of 13.2. While the double work period has the advantage of supplying more information than the single work period, our results indicate that generalization on the relative value of the two methods in the study of fatigue should be withheld pending further investigation. It may be that the explanation of the difference between our results and those of Foltz and his associates lies in the fact that our subjects were all of the athletic type and customarily took part in some form of athletic activity. Apparently they reached the peak of training in a very short time. Consideration should also be given to the possibility that the factors responsible for recovery from fatigue may not be identical with those that determine the onset of fatigue.

Muscular efficiency

The net muscular efficiency was calculated by dividing the heat equivalent of the work by the difference in the amount of heat produced during exercise and the amount produced during the same length of time in the resting state before exercise. Heat production was determined from the respiratory exchange. As shown in table 2, the net muscular efficiency was the same after both types of meals. These results are in

TABLE 2

Net muscular efficiency.[1]

SUBJECT	HIGH CARBOHYDRATE MEAL PERIODS			LOW CARBOHYDRATE MEAL PERIODS		
	First exercise		Second exercise	First exercise		Second exercise
	First 10 min.	Remainder		First 10 min.	Remainder	
	%	%	%	%	%	%
W.W.	25.7	22.7	25.4	24.6	22.6	25.7
J.L.	23.2	19.2	21.9	22.2	19.6	22.2
C.D.	22.3	20.3	22.5	22.3	20.2	22.6
J.H.	21.3	20.0	20.8	21.1	19.7	20.8
J.S.	23.9	22.9	23.6	23.8	21.3	23.3
E.H.	23.2	21.6	23.6	21.0	20.5	21.5
Average	23.3	21.1	23.0	22.5	20.7	22.7

[1] Each figure in the table is an average of 6 experiments except the final averages which are derived from 36 experiments.

agreement with those that have been reported previously (Haldi, Bachmann, Ensor and Wynn, '38). During the latter part of the first exercise period in the experiments with both the high and the low carbohydrate meals it was approximately 2% lower than in the first 10 minutes of exercise. This difference between muscular efficiency in the earlier and later stages of the first exercise period was statistically significant from which it may be concluded that fatigue is associated with a lowering of muscular efficiency. In the second period, after a short rest, the muscular efficiency was the same as in the first 10 minutes of exercise.

Blood sugar

In the resting state immediately before exercise the blood sugar level was appreciably higher after the high carbohydrate than after the low carbohydrate meal (table 3). At the conclusion of both the first and second exercise periods the blood sugar had dropped from 145 to 106 mg % in the experiments on the meal rich in carbohydrate, whereas it remained at practically the resting level in those with the low carbohydrate meal. At the end of the recovery periods the blood sugar concentration was the same in both sets of experiments.

TABLE 3

Blood sugar concentration.[1]

	HIGH CARBOHYDRATE MEAL				LOW CARBOHYDRATE MEAL			
Subject	Resting	End of first exercise period	End of second exercise period	End of recovery period	Resting	End of first exercise period	End of second exercise period	End of recovery period
	mg %	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*
W.W.	128	91	97	121	114	103	100	118
J.L.	166	116	120	119	128	137	135	125
C.D.	132	100	104	113	119	119	112	119
J.H.	130	107	108	126	123	112	112	118
J.S.	168	112	111	148	152	130	126	134
E.H.	138	112	102	109	116	119	118	113
Average	145	106	107	123	125	120	118	122

[1] Each figure in the table is an average of 6 experiments except the final averages which are derived from 36 experiments.

These data may be taken to indicate that more energy was supplied by glucose after the large than after the smaller intake of carbohydrate. In the experiments with the high carbohydrate intake the rate of glycogenolysis during exercise apparently did not keep pace with the rate of glucose utilization resulting in a drop in blood sugar from 145 to 106 mg %. Glycogenolysis during recovery appears to have proceeded faster than glucose utilization thereby raising the blood sugar level to 123 mg %. After the meal of a smaller carbo-

hydrate content glycogenolysis and glucose utilization were apparently in equilibrium.

Blood lactic acid

The lactic acid concentrations of the blood drawn at the end of the rest period immediately before exercise were 15.9 ± 5.1 and 10.0 ± 3.6 mg %, respectively, in the experiments on the high and low carbohydrate meals. The difference of 5.9 mg % must be regarded as statistically significant since it was 4 times the critical ratio. Immediately after the first and second exercise periods the blood lactic acid level was appreciably above the base line and practically the same in the 2 sets of experiments namely, 48.0 and 44.8 as compared with 46.5 and 41.4 mg %. At the conclusion of the experiment it had returned to 4 and 6 mg %, respectively, above the base level in the 2 groups of experiments.

The initial higher percentage of blood lactic acid after the meal rich in carbohydrate is believed to be due to the availability of a greater amount of glucose and fructose to the organism than after the low carbohydrate meal. It has been shown that the ingestion of both glucose and fructose lead to an increase in blood lactic acid concentration (Bachmann and Haldi, '37; Edwards, Bensley, Dill and Carpenter, '44).

Respiratory quotient

After the low carbohydrate meal the average respiratory quotient in the resting state was 0.79 (fig. 1). During the first 10 minutes of the first exercise period it rose to 0.97 and went slightly higher during the last few minutes of exercise. In the second exercise period the quotient was somewhat lower, at 0.91. These results are taken to indicate that the major portion of the energy of exercise was derived from carbohydrate but to a lesser degree in the second than in the first period. This may reasonably be assumed to be due to a reduction of the carbohydrate reserves in the first exercise period.

After the meal rich in carbohydrate the base line of the respiratory quotient was considerably higher than after the low carbohydrate meal. During the exercise and recovery periods the quotient was also at a higher level than in the experiments with the low carbohydrate meal. In the first exercise period it rose slightly above unity. Quotients of this magnitude during exercise have been reported by other investigators (Carpenter and Fox, '31). In the second 10-minute

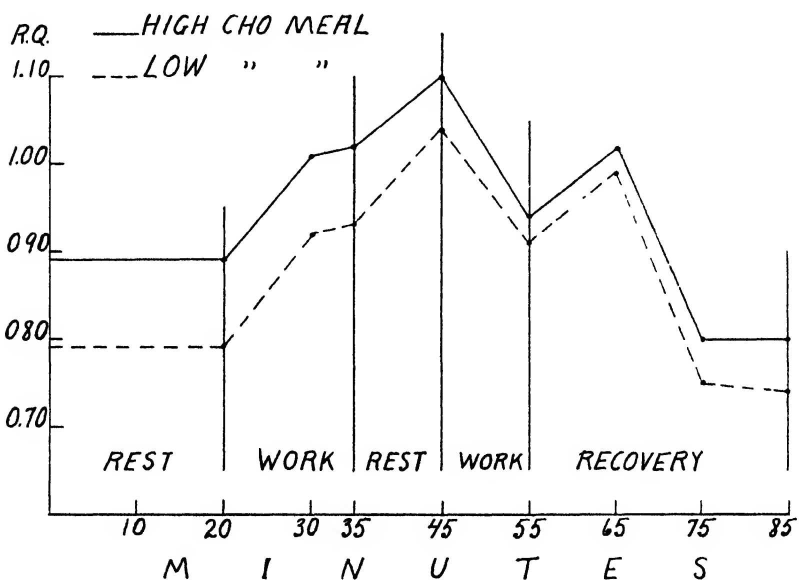


Fig. 1 The respiratory quotient during rest, exercise, and recovery after a high and a low carbohydrate meal. The 2 exercise periods in the graph are approximate averages of the length of time the subjects were able to ride the bicycle until exhausted.

period after the final exercise period the quotient descended rapidly to a level slightly below the base line and remained at this level during the last 10-minute period of the experiment. A similar drop in the quotient occurred in the experiments with a low carbohydrate meal.

From these observations and those on blood sugar it may be concluded that as a result of a larger intake of carbo-

hydrate there was more energy supplied by this food material both at rest and during exercise.

In both sets of experiments there was a marked rise in the respiratory quotient immediately after exercise which in some instances exceeded unity. This phenomenon has been observed in previous experiments of a similar nature in our laboratories (Haldi and Bachmann, '37). Analysis of the data of the former experiments led the authors to suggest that the high quotients might have been due to an acceleration of the transformation of glucose into fat by exercise which persisted a few minutes after exercise. This explanation, however, we wish to regard as only tentative until various etiological possibilities can be explored by further experimentation.

General observations

The relative work output, percentage recovery, blood sugar and muscular efficiency, after the 2 types of meals, as shown in tables 1–3, were the same regardless of age or sex.

DISCUSSION

In the treatment of the clinical syndrome of functional hypoglycemia a reduction in the carbohydrate intake has been found in a number of instances to give good results (Conn, '40). It is probably on the basis of such observations that some have been led to believe that relatively high carbohydrate meals predispose normal individuals to hypoglycemia with a concomitant feeling of weakness and a diminution in the capacity for work. The present experiments on normal healthy adolescents and adults do not support this point of view. Several hours after the ingestion of a high carbohydrate meal there was no evidence of hypoglycemia either before or immediately after severe exhausting exercise. Work output, percentage recovery, and muscular efficiency were not diminished by the relatively large carbohydrate intake. These observations afford confirmatory evidence for the conclusion drawn from previous studies (Haldi and Wynn, '46a) that

the hypoglycemic syndrome of functional origin is a pathological condition and not a normal physiological response to an antecedent high carbohydrate intake.

We are unable, at present, to account for the apparent discrepancy between our observations and those of D'Angelo ('46) who found that the ingestion of 70 or 150 gm of dextrose induced the development of "hypoglycemic" reactions in 5 out of 6 subjects approximately 50% of the total number of times tested. These reactions usually occurred 3 to 5 hours after ingestion of the sugar with approximately equal frequency at either ground level or altitude. "The seizures were characterized by subjective sensations of nervousness, impending danger, paresthesia, weakness and hunger, and objective manifestations of pallor, sweating, tremulousness, coldness of the extremities and a tendency in some toward a drop in oral temperature." It is of interest to note, however, that in most instances the blood sugar level was within the normal range at the time of the so-called hypoglycemic reaction.

None of our subjects manifested any of these symptoms. They all reported that they could observe no difference on the 2 types of meals in their subjective state or in their general physical condition at any time during the experiments. At the conclusion of each experiment they experienced a sense of weakness in the legs and knees but this soon wore off so that they were able to proceed in their usual activities without discomfort. One of them (J.H.) invariably went from the laboratory to the gymnasium where he engaged in an hour's practice in basket ball.

CONCLUSIONS

Work output, muscular efficiency, and percentage recovery (measured by a comparison of the work performance in the first and second exercise periods) were not diminished by a high carbohydrate intake 2 to 3 hours before exercise.

After the meal rich in carbohydrate the blood sugar level immediately before exercise was higher than at the corresponding time after the low carbohydrate meal.

The data on the blood sugar levels and the respiratory quotient are interpreted as indicating that a greater portion of the energy of exercise was derived from carbohydrate in the experiments with a high carbohydrate meal than after the low carbohydrate intake.

There were no age or sex differences in the comparative work performance on the 2 types of meals.

At no time throughout the experiments was there any evidence of the so-called hypoglycemic reaction to the high carbohydrate meal.

The data of these experiments are offered as confirmatory evidence for the conclusion drawn from other observations that a hypoglycemic reaction is not the usual physiological response to a high carbohydrate meal.

LITERATURE CITED

BACHMANN, G., AND J. HALDI 1937 A comparative study of the respiratory quotient following the ingestion of glucose and of fructose as affected by the lactic acid and carbon dioxide changes in the blood. J. Nutrition, *13:* 157.

BARKER, S. B., AND W. H. SUMMERSON 1941 The colorimetric determination of lactic acid in biological material. J. Biol. Chem., *138:* 535.

CARPENTER, THORNE M., AND E. L. FOX 1931 The effect of muscular work upon the respiratory exchange of man after ingestion of glucose and of fructose. I. The respiratory quotient and the mechanics of breathing. Arbeitsphysiol., *4:* 532.

CONN, J. W. 1940 The spontaneous hypoglycemias. Importance of etiology in determining treatment. J. Am. Med. Asso., *115:* 1669.

D'ANGELO, S. 1946 Blood sugar levels and carbohydrate administration in human subjects during prolonged exposures to moderately low altitudes. Am. J. Physiol., *145:* 365.

EDWARDS, H. T., E. H. BENSLEY, D. B. DILL AND T. M. CARPENTER 1944 Human respiratory quotients in relation to alveolar carbon dioxide and blood lactic acid after ingestion of glucose, fructose or galactose. J. Nutrition, *27:* 241.

FOLTZ, E., A. C. IVY AND C. J. BARBORKA 1942 The use of double work periods in the study of fatigue and the influence of caffeine on recovery. Am. J. Physiol., *136:* 79.

HALDI, J., AND G. BACHMANN 1937 The effect of exercise on metabolism following the ingestion of water, glucose and fructose as shown by the course of the respiratory quotient. J. Nutrition, *14:* 287.

HALDI, J., G. BACHMANN, C. ENSOR AND W. WYNN 1938 Muscular efficiency in relation to the taking of food and to the height of the respiratory quotient immediately before exercise. Am. J. Physiol., *121:* 123.

HALDI, J., AND W. WYNN 1946a The effect of low and high carbohydrate meals on the blood sugar level and on work performance in strenuous exercise of short duration. Am. J. Physiol., *145:* 402.

———— 1946b Observations on efficiency of swimmers as related to some changes in pre-exercise nutriment. J. Nutrition, *31:* 525.

PETERS, J. P., AND D. D. VAN SLYKE 1932 Quantitative Clinical Chemistry, vol. 2, Methods. Williams and Wilkins Co., Baltimore.

THORNE, G. W., J. T. QUINBY AND M. CLINTON 1943 A comparison of the metabolic effects of isocaloric meals of varying composition with special reference to the prevention of postprandial hypoglycemic symptoms. Ann. Int. Med., *18:* 913.

WILDER, R. M. 1943 In Symposium on nutrition in industry. J. Am. Med. Asso., *121:* 868.

IMPROVING THE NUTRITIVE VALUE OF FLOUR

I. THE EFFECT OF SUPPLEMENTING ENRICHED FLOUR WITH OTHER B-COMPLEX VITAMINS [1]

BEULAH D. WESTERMAN AND GERTRUDE HALL

Department of Food Economics and Nutrition, Kansas Agricultural Experiment Station, Kansas State College, Manhattan

FOUR FIGURES

(Received for publication August 26, 1946)

There is a great demand for cereal products by much of the world's population. A large proportion of the food calories of many people is contributed by bread and cereals. Stiebling, Monroe, Coons, Phipard and Clark ('41) found that grain products furnished 25% of the total calories in the diets of farm families in the northern and western parts of the United States and 38% in the southeastern part. Data collected from government publications by Cummings ('40) showed that bread, flour and cereals provided 19% of the calories in the diets of families of professional men, 27% in well-to-do farm families and 54% in poor farm families in the southern states. Holman ('46) summarized the situation by stating that the proportion of cereals, in diets commonly used, varies from less than 30% of the total in the British and North American diets to 80–90% in the oriental diets.

In recent years, investigations regarding flour and bread have been stimulated by the demand for improvement in the nutritive value of these products. Bread and cereals are important sources of carbohydrates and protein. The proper

[1] Contribution no. 134 of the Department of Home Economics, Kansas Agricultural Experiment Station, Manhattan.

oxidation of the carbohydrates in animal metabolism is dependent upon the presence of a definite quantity of the B-complex vitamins in the diet. Because of this fact it was considered advisable to continue the work on improving the nutritive value of flour by the addition of B-complex vitamins. The literature was reviewed in a previous paper by Westerman and Bayfield ('45). The present paper presents further information regarding this problem. The agents which have been chosen for study are calcium pantothenate, pyridoxine, choline, and the addition of greater quantities of riboflavin and thiamine. At a time when good food sources of the B-complex vitamins such as meat and milk are apt to be low or lacking in the diets of many people, it seems logical that the addition of synthetic vitamins to flour may be a method of taking care of this deficiency.

EXPERIMENTAL PROCEDURE AND RESULTS

The experimental procedure followed that described in the paper by Westerman and Bayfield ('45). Young albino rats weighing between 40–50 gm were used in the 4 experiments reported here. Table 1 gives the detailed composition of the diets.

First experiment: flour and wheat as 50% of the diet

In the first experiment the flour and wheat were fed at a level of 50% in the diet and replaced an equivalent amount of sucrose. The 50% level was chosen because before the war people in many European countries and certain low income groups in the United States consumed diets containing that much or more of cereal foods. The flour was milled from the same lot of wheat that was used in the test. Thiamine, riboflavin and calcium pantothenate assays on the wheat showed 4.1, 1.1 and 7.7 µg per gm, respectively. The pyridoxine content was not determined but Swaminathan ('40) reported 7 µg pyridoxine per gm in wheat and Tepley, Strong and Elvehjem ('42) reported a 50% loss in the milling process.

The rats ate on the average 6 gm of food daily. Since the diet was 50% wheat or flour they consumed approximately 3 gm of these each day. Therefore, the wheat in the diet supplied 12.3 μg of thiamine, 3.2 μg of riboflavin, 23.1 μg calcium pantothenate, and perhaps 21 μg of pyridoxine daily. The flour assayed 4.7, 3.1 and 2.6 μg for thiamine, riboflavin and calcium pantothenate, respectively, and supplied the animals with 14.1 μg thiamine, 9.4 μg riboflavin, 7.7 μg calcium pantothenate and perhaps 10.5 μg pyridoxine daily. Diets IV and V had 10.3 μg of calcium pantothenate added per gm of flour thereby bringing the total daily intake of calcium pantothenate up to 38.6 μg. Diet V had 3.5 μg of pyridoxine per gm added to the flour making the probable daily intake of pyridoxine 21 μg.

After the animals were placed on the test materials, they were weighed every 6 days. The growth test was conducted over a period of 72 days. The average weight gains of each group are shown in figure 1.

Those animals on the B-complex free diet lost weight rapidly and died in about 18 days. Those on ground whole wheat gained on the average 27 gm during the 72-day test, while those on enriched flour gained an average of 30 gm. The animals on enriched flour to which calcium pantothenate was added made an average gain of 44 gm during the test while those with both calcium pantothenate and pyridoxine added showed an average gain of 101 gm. The animals on the stock diet made an average gain of 190 gm during the test.

When 10.3 μg calcium pantothenate per gm were added to the enriched flour, the increase in growth was above that of the groups on enriched flour or whole wheat. This seems to indicate that more calcium pantothenate was needed for growth than that furnished by the enriched flour or the whole wheat. When both 10.3 μg calcium pantothenate and 3.5 μg pyridoxine per gm were added, much better growth was noted, apparently showing a need for both of these vitamins. However, with the vitamins added at the levels indicated, growth was not as good as that of the animals on the stock diet. It

TABLE 1

Composition of the diets.

The basal vitamin B-complex free diet consisted of 20% vitamin free casein, 60% sucrose, 12% fat, 5% salt mixture and 3% cod liver oil. In experiment I the flour and wheat were fed at a 50% level in the diet and replaced an equivalent amount of sucrose. In experiments II, III, and IV 40% of the diet was enriched flour with the B vitamins added to the flour as indicated below.

DIET NO.	EXPERIMENT I Changes in diet	EXPERIMENT II Changes in diet	EXPERIMENT III Changes in diet	EXPERIMENT IV Changes in diet
I	Basal diet	40% enriched flour	40% enriched flour	40% enriched flour
II	50% enriched flour	Added pyridoxine (3.5 μg/gm)	Added pyridoxine (3.5 μg/gm)	Added riboflavin (1 μg/gm)
III	50% ground whole wheat	Added pyridoxine (7 μg/gm)	Added pyridoxine (7 μg/gm)	Added riboflavin (1 μg/gm); and thiamine (1 μg/gm)
IV	Enriched flour + Ca pantothenate (10.3 μg/gm)	Added Ca pantothenate (10.3 μg/gm)	Added Ca pantothenate (10.3 μg/gm)	Added pyridoxine (14 μg/gm)
V	Added Ca pantothenate (10.3 μg/gm); and pyridoxine (3.5 μg/gm)	Added Ca pantothenate (20.5 μg/gm)	Added Ca pantothenate (20.5 μg/gm)	Added choline (3 mg/gm); and pyridoxine (7 μg/gm)
VI	Stock diet	Added pyridoxine (3.5 μg/gm); and Ca pantothenate (10.3 μg/gm)	Added pyridoxine (3.5 μg/gm); and Ca pantothenate (10.3 μg/gm)	Added pyridoxine (14 μg/gm); and Ca pantothenate (40 μg/gm)
VII		Added pyridoxine (7 μg/gm); and Ca pantothenate (20.5 μg/gm)	Added pyridoxine (7 μg/gm); and Ca pantothenate (20.5 μg/gm)	Added Ca pantothenate (40 μg/gm)
VIII		Stock diet		Added choline (3 mg/gm); and Ca pantothenate (20 μg/gm)
IX				Added pyridoxine (14 μg/gm); Ca pantothenate (40 μg/gm); and choline (3 mg/gm)

appears that other factors are involved, perhaps a need for greater quantities of the B-complex vitamins already added or other members of the B-complex group.

At the end of 72 days, the males and the females on the same diet were placed together for breeding purposes. The animals on the stock diet produced normal litters. Of those on the experimental diets, only the animals with calcium pantothenate and pyridoxine added to the flour produced any young. Only half of the females on this diet had young, and these died before maturity.

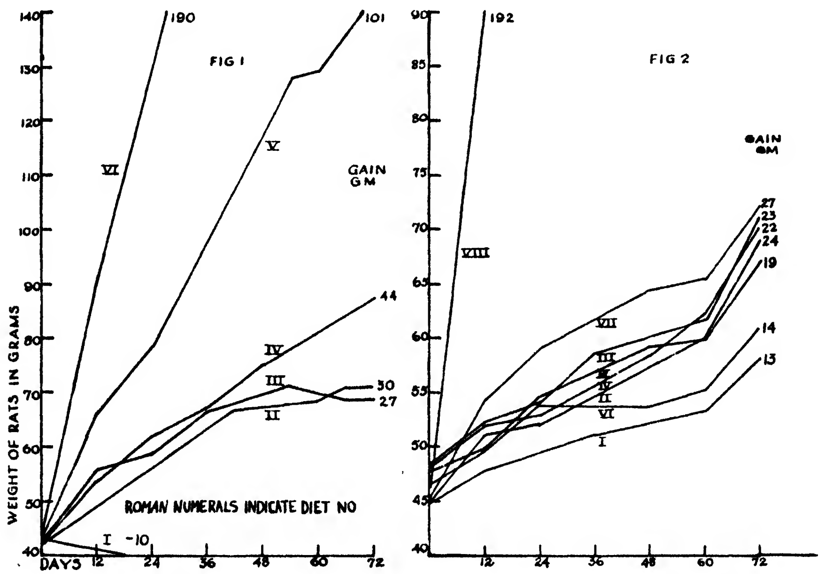


Fig. 1 Growth curves of animals in Experiment I.
Fig. 2 Growth curves of animals in Experiment II.

Second experiment: effect of increasing calcium pantothenate and pyridoxine, with flour as 40% of the diet

In an attempt to obtain better growth and reproduction in the animals, a second experiment was conducted to determine the effect of increasing the quantities of calcium pantothenate and pyridoxine. The amount of flour used in the diet

was 40%. This change was made because it was thought that the American people might consume cereals at a 40% level in the diet but it was doubtful that the 50% level would be reached by a large percentage of the population. The flour was a good grade of enriched flour purchased from the grocer. Analyses showed that it contained 5.8 μg per gm of thiamine, 2.6 μg per gm of riboflavin and 2.0 μg per gm of calcium pantothenate.

There were 8 groups of animals. The composition of the diets is given in table 1. The test covered a 72-day period with the results as shown in figure 2.

On the average, the animals did not gain as much as those in the previous test with the same enriching materials. This may partially be explained by the change from 50 to 40% in the quantity of flour in the diet. Since the flour carried the B-complex vitamins, the quantities of these were therefore decreased.

The growth curves (fig. 2) show that the animals on the enriched flour, diet I, made the least gains. They gained 13 gm during the 72-day test period. Those animals on diet VI with 3.5 μg pyridoxine and 10.3 μg calcium pantothenate per gm added to the enriched flour grew rather rapidly for the first 24 days of the test, then barely maintained their weight until the sixtieth day when they started to gain again, but their total gain was only 14 gm during the test. The animals on diets II, III, IV and V made approximately the same gains throughout the test. Those on diet II, with 3.5 μg additional pyridoxine per gm of flour, showed greater gains during the last 12 days on the test, but as 4 of these animals died, it may be that the stronger ones survived, thus accounting for the greater gain. The rats on diet VII, with 7 μg pyridoxine and 20.5 μg calcium pantothenate added per gm of flour, showed better average gains throughout the test, with a total average gain of 27 gm.

Increasing the amount of pyridoxine alone did not increase the growth rate. The animals on 3.5 μg pyridoxine made an average gain of 24 gm, while those on 7 μg pyridoxine per gm

made an average gain of 23 gm. Increasing the calcium pantothenate did not increase growth. Animals on 10.3 μg per gm gained on the average 22 gm and those on 20.5 μg per gm gained 19 gm. When both the pyridoxine and calcium pantothenate were increased, there was an increase in weight of 13 gm over the animals receiving 3.5 μg pyridoxine and 10.3 μg calcium pantothenate per gm of flour.

The animals on this test were mated for breeding purposes, but no young were born. Then the females were placed with normal males but kept on the same experimental diets. Those on enriched flour alone did not reproduce, those with 3.5 μg pyridoxine per gm added to the flour produced litters, but all the young died or were eaten by the mother within 2 days after birth. The females receiving the 7 μg pyridoxine per gm of flour did not reproduce; this observation was unexpected and is unexplainable. The females on the diet with flour plus calcium pantothenate alone did not produce young while those with flour plus both pyridoxine and calcium pantothenate produced young; however, all the young died within 7 days.

Third experiment: repetition of second experiment with longer feeding period

In the third experiment (fig. 3), 8 animals were used in each series and the diets contained 40% enriched flour in which the added vitamins were incorporated. This test was a partial repetition of the second experiment, but was continued for 90 days. On the average, the animals on this test made better gains than in the previous test. The greatest gains (average 73 gm) were made by the animals on diet VII, which had 7 μg pyridoxine and 20.5 μg calcium pantothenate per gm added to the flour. These were followed by the animals on diet VI, with 3.5 μg pyridoxine and 10.3 μg calcium pantothenate per gm, and also those on diet III, with 7 μg of added pyridoxine with average gains of 56 and 57 gm, respectively. However the curves show that the animals on diet III always gained less than those on diet VI. There was only a slight difference

in the average weight gain of 47 gm by the group on diet V with 20.5 μg calcium pantothenate and the 45 gm average weight gain by those animals on diet II with 3.5 μg pyridoxine, while those on diet IV with 10.3 μg calcium pantothenate per gm made an average weight gain of 42 gm. The animals on enriched flour alone made the least average gain, 38 gm, during the test.

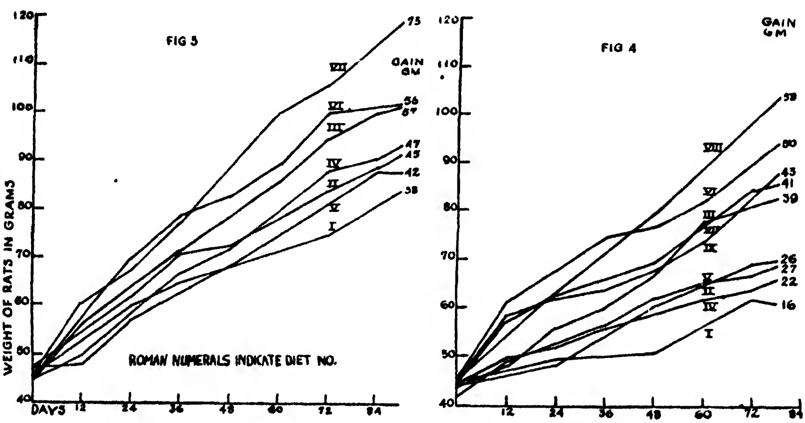


Fig. 3 Growth curves of rats in Experiment III.
Fig. 4 Growth curves of rats in Experiment IV.

Over the 90-day period, it seems that increasing both the pyridoxine and calcium pantothenate had a beneficial result. Increasing the calcium pantothenate alone had some beneficial results while increasing the pyridoxine alone seemed to exert a greater stimulus on growth than did calcium pantothenate.

Fourth experiment: effect of increasing riboflavin and thiamine as well as calcium pantothenate, pyridoxine, and of adding choline

In the fourth experiment, it was decided to test the effect of increasing the amounts of riboflavin and thiamine in the flour each by 1 μg per gm of flour, and also to test both the effect of adding much larger amounts of calcium pantothenate

and pyridoxine and that of adding choline. Table 1 shows the composition of the diets. The test covered a period of 78 days.

The animals on enriched flour alone made the least gain. Their average weight gain during the test was 16 gm. The addition of riboflavin increased the gain to 26 gm, while the addition of both riboflavin and thiamine caused a gain of 39 gm. This was a slightly greater gain than was made by the animals with pyridoxine added or pyridoxine and choline, which made gains of 22 and 27 gm, respectively. The animals with pyridoxine, calcium pantothenate, and choline made approximately the same gain, 43 gm, as those on 40 μg calcium pantothenate per gm which gained 41 gm. The rats receiving 14 μg pyridoxine and 40 μg calcium pantothenate per gm gained 50 gm. This was not as much of a gain as was exhibited by the rats that received choline and 20 μg calcium pantothenate per gm of flour, which made an average gain of 58 gm. The effect of the addition of choline to the enriched flour appeared to be slight, but this might have been expected since the diet contained 20% casein. In all probability the methionine in the casein provided sufficient methyl groups for the formation of choline.

DISCUSSION

Flour enriched at the levels recommended by the National Research Council with thiamine, riboflavin and nicotinic acid, when used as the sole source of the B-complex vitamins does not supply enough of these vitamins to support normal growth in rats when fed at a level of 40 or 50% in the diet. While flour and bread are not necessarily expected to supply all the B-complex vitamins in the diet, it might be well to have more of these vitamins supplied by the cereal foods inasmuch as cereals make up a major portion of the food intake of so many people in the world. There is the added factor of a lack of adequate supply of many of the foods which carry the B-complex vitamins, particularly meat and milk. When milk is available it is used in the making of bread, thereby adding not only thiamine, riboflavin and nicotinic acid but other B vita-

mins. At present it may be questioned whether there is enough milk available for this purpose in the United States; it is certainly not in the rest of the world.

In a report by Milan and Bell ('46) regarding the nutrients in the diets of a North Carolina village population it is stated that pork and milk contributed, respectively, 14.3% and 11.4% of the thiamine in the adult diets while enriched bread contributed 16.7% of the thiamine and dried peas and beans furnished 19.6%. As sources of riboflavin in these diets milk ranked first, contributing 42.2% and eggs second with 11.2%. Enriched bread contributed only 5.2% of the riboflavin in the total diet.

There is the possibility that enriched bread might contribute more riboflavin to the diet. In our experiments it was found that the addition of even a small quantity of riboflavin to the enriched flour increased the rate of growth while the addition of both riboflavin and thiamine gave a still greater growth increase. In all the experiments the addition of pyridoxine or calcium pantothenate alone increased the growth rate but when they were added together still better growth was obtained.

Supplee, Bender and Kahlenberg ('42) found that 20 µg of calcium pantothenate per day were adequate for normal rat growth. In our experiments the animals ate each day, on the average, 6 gm of food which contained 2.4 gm of flour. The flour with 10.3 µg added calcium pantothenate per gm increased the calcium pantothenate in the diet by 24.7 µg. According to Supplee et al. ('42), this was an ample amount of the vitamin. Our data appear to confirm this since increasing the calcium pantothenate in the flour above 10.3 µg per gm did not result in a corresponding weight increase (figs. 2 and 3).

The addition of pyridoxine and calcium pantothenate to enriched flour under the conditions of these experiments appears to have a beneficial effect on the growth rate of the rat. Increasing the added pyridoxine from 3.5 µg per gm to 7 µg per gm in the flour produced increased growth (fig. 3). When both calcium pantothenate and pyridoxine were in-

creased from 10.3 μg per gm to 20.5 μg per gm and from 3.5 μg per gm to 7 μg, respectively, there was an increased growth as shown in figures 2 and 3. Lepkovsky and Nielson ('42) reported a relationship of pyridoxine to tryptophane metabolism in rats. They suggest that normal tryptophane metabolism is regulated by pyridoxine. That pyridoxine is necessary for the formation of the coenzyme which functions in the tyrosine decarboxylation reaction of certain bacteria has been shown by Gunsalus, Bellamy and Umbriet ('44). Follis and Wintrobe ('45) find that a deficiency of pyridoxine and pantothenic acid in the diet of swine resulted in degeneration of the sensory neurons. While it has not been definitely proven that pyridoxine and pantothenic acid are essential in the diet of humans, experiments on other animals indicate that these vitamins have a function in animal metabolism.

Sure ('41) noted abnormalities in reproduction when calcium pantothenate was lacking in the diet. Nelson and Evans ('46) found that a deficiency of pantothenic acid in the diet on or before the day of mating always resulted in failure to reproduce or in abnormal young. The results reported in the present paper are in line with those of these investigators since offspring were obtained only from the mothers who received calcium pantothenate in the diet and the litters were defective and did not mature.

The quantities of pyridoxine and calcium pantothenate along with the other B-complex vitamins to be added to enriched flour in order to produce normal growth and normal young require further study and this work is being continued.

SUMMARY

Evidence has been presented to show that the supplementing of enriched flour with certain B-complex vitamins improves the nutritive value of the flour. Under the conditions of the experiments the addition of 1 μg of riboflavin per gm of enriched flour produced an increase in the growth rate of the animals. The addition of 1 μg of riboflavin and 1 μg of thiamine to the enriched flour stimulated even greater growth of

the rats. The addition to 10.3 μg calcium pantothenate and 3.5 μg pyridoxine per gm of enriched flour was beneficial, but a still greater increase in growth resulted when 20.5 μg calcium pantothenate and 7 μg pyridoxine per gm were added to the enriched flour.

LITERATURE CITED

CUMMINGS, R. O. 1940 The American and His Food. Univ. of Chicago Press, Chicago, Ill.

FOLLIS, RICHARD H., JR., AND MAXWELL M. WINTROBE 1945 A comparison of the effects of pyridoxine and pantothenic acid deficiencies on the nervous tissues of swine. J. Exp. Med., *81:* 539.

GUNSALUS, I. C., W. D. BELLAMY AND W. W. UMBREIT 1944 A phosphorylated derivative of pyridoxol as the coenzyme of tyrosine decarboxylase. J. Biol. Chem., *155:* 685.

HOLMAN, W. I. M. 1946 The amounts of vitamin B_1 in cereals and the extent to which they supply human requirements in various dietaries. Nutr. Abst. and Rev., *15:* 387.

LEPKOVSKY, SAMUEL, AND ELMER NIELSON 1942 A green pigment-producing compound in urine of pyridoxine-deficient rats. J. Biol. Chem., *144:* 135.

MILAN, D. F., AND MARY PARKS BELL 1946 Sources of principal nutrients in the diet of a North Carolina village population. J. Am. Diet. Assn., *22:* 411.

NELSON, MARJORIE M., AND HERBERT M. EVANS 1946 Pantothenic acid deficiency and reproduction in the rat. J. Nutrition, *31:* 497.

STIEBLING, H. K., D. MONROE, C. M. COONS, E. F. PHIPARD AND F. CLARK 1941 Family food consumption and dietary levels. U. S. Dept. Agric. Misc. Publ. no. 405.

SUPPLEE, G. C., R. C. BENDER AND O. J. KAHLENBERG 1942 Interrelated vitamin requirements: Kidney damage, adrenal hemorrhage and cardiac failure correlated with inadequacy of pantothenic acid. Endocrinology, *30:* 355.

SURE, B. 1941 Dietary requirements for fertility and lactation. XXIX. The existence of a new dietary factor essential for lactation. J. Nutrition, *22:* 499.

SWAMINATHAN, M. 1940 Chemical estimation of vitamin B_6 in foods by means of the diazo reaction and the phenol reagent. Nature, *145:* 780.

TEPLEY, L. J., F. M. STRONG AND C. A. ELVEHJEM 1942 Nicotinic acid, pantothenic acid and pyridoxine in wheat and wheat products. J. Nutrition, *24:* 167.

WESTERMAN, B. D., AND E. G. BAYFIELD 1945 Enriched, Morris type and whole wheat flour as sources of the B-complex vitamins. J. Nutrition, *29:* 27.

AMINO ACIDS REQUIRED TO SUPPLEMENT LINSEED PROTEIN FOR CHICK GROWTH

F. H. KRATZER, DELBERT WILLIAMS AND ELIZABETH F. BAKER
Division of Poultry Husbandry, University of California, Davis

(Received for publication October 28, 1946)

Linseed meal has been shown to be detrimental to growing chicks when it is fed at levels of 4.5% or more of the ration (Bethke et al., '28; Ackerson et al., '38; Sherwood and Couch, '40; Slinger et al., '43; Hammond, '44; Heuser and Norris, '44; Heuser et al., '46). Since its nutritional value may be increased by a simple water treatment (Kratzer, '46; McGinnis and Polis, '46), apparently the detrimental action of linseed meal depends upon a toxic substance, not upon a serious amino acid deficiency in the protein. By using water-treated linseed meal as the sole source of protein in a chick ration, one can determine the adequacy of the protein in meeting the amino acid requirement of chicks without the interference of the toxic factor.

EXPERIMENTAL PROCEDURE

Commercial linseed meal was treated with water for 24 hours and dried according to the procedure outlined previously (Kratzer, '46). The composition of the basal diet in which the treated linseed meal was fed is given in table 1. The crude protein content of the diet was not determined for the first experiment, but in the second and third experiments the rations were found to contain 18.5 and 20.3%, respectively.

Day-old S. C. White Leghorn chicks were fed a practical starting ration for 11 days. At the end of this period they were divided into comparable groups of 8 chicks each and were fed the experimental rations ad libitum. They were housed in electrically heated batteries with wire floors. The trials were continued for 9, 8, and 7 days, respectively, in successive

TABLE 1

Minerals and vitamins contained in the basal diet.

BASAL MIXTURE	
Treated linseed meal:	
Trials 1 and 2	58 gm
Trial 3	61 gm
Minerals, vitamins and other organic supplements . . . as indicated below	
Glucose: to bring the total up to	100 gm

MINERAL SUPPLEMENTS	gm	VITAMINS AND OTHER ORGANIC SUPPLEMENTS	gm
Calcium gluconate	5.0	Soybean oil	3.0
Tricalcium phosphate	2.0	Fish oil (3000 A–400 D)	0.5
Dicalcium phosphate	1.5	Choline chloride	0.2
Sodium chloride	1.0	Cholic acid	0.1
Dipotassium phosphate	0.5	Inositol	0.1
Magnesium sulfate	0.3	Niacin	0.01
Potassium chloride	0.3	dl-alpha tocopherol acetate	0.001
Sodium silicate	0.2	Calcium pantothenate	0.001
Manganese sulfate	0.03	2-methyl-1, 4-naphthoquinone	0.001
Ferric oxide	0.02	Riboflavin	0.0005
Aluminum sulfate	0.025	Thiamine	0.0005
Copper sulfate	0.005	Pyridoxine	0.0004
Zinc sulfate	0.005	Folic acid	0.0001
Cobalt acetate	0.002	Biotin	0.00001
Potassium iodide	0.001		

experiments. With the exception of 1 chick which had been fed the basal ration with no amino acid supplements in the third experiment, there was no mortality. One control group fed stock mash was included in each experiment.

Amino acids were added to the basal ration as indicated in table 2. Those used were commercial preparations in the following forms: l (+)-arginine monohydrochloride, l(—)-

cystine, l(+)-lysine monohydrochloride, dl-methionine, and dl-tryptophane.

The growth data were calculated as per cent gain per day according to the following formula:

$$\frac{\text{total gain} \times 100}{\text{average body weight} \times \text{number of days}} = \text{per cent gain per day}$$

This criterion permits a better comparison of data from different experiments than is possible by the use of average gains.

TABLE 2

Growth of chicks fed a basal ration containing linseed protein supplemented with various amino acids.

SUPPLEMENT	LEVEL IN PER CENT OF RATION	GAIN BODY WEIGHT (% GAIN PER DAY)		
		Experiment 1	Experiment 2	Experiment 3
None		5.1	5.1	4.9
Arginine	0.2	5.1	5.5	
Lysine	0.2	6.5		
Lysine	0.4		7.0	7.1
Lysine	0.5	6.9		
Methionine	0.2	5.1	5.0	
Cystine	0.162	5.6	5.5	
Tryptophane	0.1	5.6	5.3	
Glycine	0.5	5.9	5.4	
Lysine	0.5			
Methionine	0.2	7.2		
Lysine	0.4			
Methionine	0.2		7.0	7.1
Lysine	0.5			
Cystine	0.162	6.7		
Lysine	0.4			
Glycine	0.5			7.4
Lysine	0.4			
Methionine	0.2		8.3	7.2
Glycine	0.5			
Arginine	0.2			
Lysine	0.4 [1]			
Methionine	0.2	7.2	8.3	
Cystine	0.162			
Tryptophane	0.2			
Glycine	0.5			
Stock Mash		7.4	8.3	6.8

[1] Lysine fed at 0.5% in experiment 1.

RESULTS AND DISCUSSION

As table 2 shows, in the first experiment lysine produced a marked growth response, while the addition of the other amino acids did not give any appreciable increase in the growth rate. The combination of lysine and methionine was slightly better than lysine alone and was as satisfactory as the combination of the 6 amino acids tested. Since 0.5% lysine caused an increase in growth above 0.2% lysine, evidently the lower level does not adequately supplement linseed protein.

In the second experiment, lysine again proved to be the only amino acid to cause an appreciable increase in growth rate. The addition of glycine to the ration containing lysine and methionine, however, caused growth to increase from 7.0% gain per day to 8.3%. Since the crude protein of the diet used in this experiment was suboptimal, conceivably the addition of even a dispensable amino acid might stimulate a growth increase.

With the crude protein level raised to 20.3% in the third experiment, lysine supplementation resulted in a growth rate of 7.1% per day, whereas the combination of lysine, methionine, and glycine caused a growth rate of 7.2% per day. Methionine added to the ration containing lysine alone caused no increase in growth; and when it was added to the ration containing both lysine and glycine, there was a reduction of 0.2% per day. When glycine was added to a ration containing lysine, growth was increased by 0.3% per day; and when glycine was added to a ration containing lysine and methionine, the increase was 0.1% per day. For the chick, therefore, linseed protein is apparently adequate in methionine. Glycine is probably adequate, although slight gains of doubtful significance were observed after its addition. Lysine constitutes the principal amino acid deficiency in linseed protein.

Block and Bolling ('45) have found that linseed protein contains the following percentages of amino acids: arginine, 6.2; lysine, 2.5; methionine, 3.0; cystine, 1.9; and tryptophane, 1.9. For a protein to be complete for the chick with respect to these amino acids (Almquist, '42; Grau and Almquist, '43,

'44), it should contain arginine, 4.5%; lysine, 4.5%; methionine, 2.5%; cystine, 1.5%; and tryptophane, 1.0%. According to these values, lysine is the only amino acid in which one would expect linseed protein to be deficient for producing rapid growth of the chick.

Since linseed meal supplied all the protein in these tests, rather than protein that is merely supplementary to the cereal proteins of a practical ration, the level of linseed meal was greater than would ever be used in a practical ration. In every experiment, a diet containing linseed protein properly supplemented with amino acids produced as good growth as a practical chick starting ration. Thus the water treatment applied to the meal seems to have been entirely effective in destroying the growth inhibitor, and the principal limitation in the practical use of linseed protein lies in its lysine deficiency.

SUMMARY

1. Linseed meal treated with water to destroy its toxic principle was fed to chicks as the sole source of protein.

2. Lysine is the principal amino acid deficiency in linseed protein.

ACKNOWLEDGMENTS

We are indebted to the following for donations of materials used in this work: Hoffmann-LaRoche, Inc., Nutley, N. J., for dl-alpha tocopherol; Merck and Company, Rahway, N. J., for synthetic biotin; and Lederle Laboratories, Inc., Pearl River, N. Y., for synthetic folic acid.

LITERATURE CITED

ACKERSON, C. W., M. J. BLISH AND F. E. MUSSEHL 1938 The utilization of food elements by growing chicks. V. A comparison of cottonseed meal and linseed oil meal as portions of the protein concentrate. Univ. Nebraska Agr. Exp. Sta. Bull. 100.

ALMQUIST, H. J. 1942 The amino acid requirements and protein metabolism of the avian organism. Fed. Proc., *1:* 269.

BETHKE, R. M., G. BOHSTEDT, H. L. SASSAMAN, D. C. KENNARD AND B. H. EDDINGTON 1928 The comparative nutritive value of the proteins of linseed meal and cottonseed meal for different animals. J. Agr. Res., *36:* 855.

BLOCK, R. J., AND D. B. BOLLING 1945 The Amino Acid Composition of Proteins and Foods. Charles C. Thomas, Springfield, Ill.

GRAU, C. R., AND H. J. ALMQUIST 1943 The utilization of the sulfur amino acids by the chick. J. Nutrition, *26:* 631.

———— 1944 Requirement of tryptophane by the chick. J. Nutrition, *28:* 263.

HAMMOND, J. C. 1944 Partial substitutes for soybean meal. Poult. Sci., *23:* 78.

HEUSER, G. F., AND L. C. NORRIS 1944 Soybean oil meal in chick rations. Cornell Bull. 810.

HEUSER, G. F., L. C. NORRIS AND J. MCGINNIS 1946 Vegetable protein concentrates fed alone and in combination with soybean oil meal and fish meal as the chief supplementary protein in chick starting rations. Poult. Sci., *25:* 130.

KRATZER, F. H. 1946 The treatment of linseed meal to improve its feeding value for chicks. Poult. Sci., *25:* 541.

MCGINNIS, J., AND H. L. POLIS 1946 Factors affecting the nutritive value of linseed meal for growing chicks. Poult. Sci., *25:* 408. (Abstract.)

SHERWOOD, R. M., AND J. R. COUCH 1940 Values of various protein feeds for growing chicks. Texas Agr. Exp. Sta. Bull. 588.

SLINGER, S. J., J. C. SMALL, I. MOTZOK AND F. N. MARCELLUS 1943 Linseed oil meal replacing meat meal in rations for growing chicks. Sci. Agr., *23:* 732.

AN INBORN CHARACTERISTIC DETERMINING THE RESPONSE OF CHICKENS TO A DIET FREE OF ANIMAL PROTEIN

H. R. BIRD, MAX RUBIN AND A. C. GROSCHKE
Bureau of Animal Industry, Agricultural Research Center, Beltsville, Maryland

TWO FIGURES

(Received for publication September 23, 1946)

It was reported by Rubin and Bird ('46) that the feeding of concentrates prepared from cow manure stimulated the growth of chicks whose basal diet consisted largely of corn and soybean oil meal. The assaying of such concentrates was greatly facilitated by using the progeny of hens fed a similar deficient diet, since hens whose diets contained the growth-stimulating factor readily transmitted it to their chicks.

Further investigation has revealed that the response of chicks to the diet being studied is influenced not only by the diet of their dams but also by some unknown characteristic of the dams which is highly variable among the population studied. This variability is manifested in hatchability of eggs and in viability and growth of progeny. The present report is concerned with this variability which is of theoretical interest for its own sake and of practical interest because of its undesirable effect on the study of diets and dietary supplements.

METHODS AND RESULTS

Variation in response of hens to a diet inadequate from the standpoint of hatchability

The experimental diets used both in growth and in hatchability studies are summarized in table 1. They are identical

with the diets used in previously reported experiments (Rubin and Bird, '46; Bird et al., '46).

The hens were yearling crossbreds (Rhode Island Red × Barred Rock) and were mated to New Hampshire males. Just prior to the initiation of these studies they had been used in a hatchability experiment of approximately 10 months' duration and had been fed the basal diet or a slightly modified diet

TABLE 1

Composition of diets.

INGREDIENTS	GROWTH STUDIES	HATCHABILITY STUDIES	
	Basal mixture	Basal mixture	Positive control
	%	%	%
Ground yellow corn	38	57	78.3
Ground barley	20		
Alfalfa meal	3	5	5
Soybean meal	35	30	
Sardine meal			10
Butyl fermentation solubles (250 μg riboflavin/gm)	0.6	0.5	0.5
Limestone flour	1.0	2.3	2.0
Steamed bonemeal	1.5	4.2	3.2
Salt (96% NaCl, 4% Mn SO_4)	0.7		
Salt (94% NaCl, 6% Mn SO_4)		0.5	0.5
Iodized salt		0.2	0.2
Vitamin A and D oil [1]	0.2	0.3	0.3
Total	100.0	100.0	100.0
Added to the above			
Choline chloride	0.05		
Nicotinic acid hydrochloride	0.001		

[1] 2000 U.S.P. units of vitamin A and 400 A.O.A.C. units of vitamin D per gm.

similar in its effect on hatchability (Bird et al., '46). Some of the data accumulated in that experiment were used in the present investigation.

Individual hatchability records were calculated for 183 hens which had been housed in laying houses and fed the basal diet or a modification similar in its effect on hatchability. Individual records covering a period of 6 months were also calcu-

lated for 67 hens of the same breeding kept in laying cages on wire screen floors and fed the basal diet. All eggs were incubated except those produced during 4 non-consecutive weeks. Table 2 shows the distribution of the hens into groups characterized by low, intermediate, and high hatchability. The birds kept on wire floors were treated separately because it had been shown that cow manure fed with this basal diet was highly beneficial to hatchability, and it was thought that coprophagy might explain the superior performance of some of the birds kept in laying houses. However, the distribution of the hens kept on wire floors was not much different from that of the others and coprophagy would not appear to be a factor. It is surprising to note that a number of individuals

TABLE 2

Distribution of hens fed deficient diet according to hatchability of their eggs and type of cage floor.

HATCHABILITY RANGE IN % OF FERTILE EGGS	HENS ON CONCRETE FLOORS COVERED WITH LITTER		HENS ON WIRE FLOORS	
	No. of hens	% of total	No. of hens	% of total
0–70	80	44	33	49
70–85	68	37	17	25
85–100	35	19	17	25

maintained excellent hatchability during a period of 10 months although they received a diet that was quite inadequate for the majority.

It seemed possible that this variability might be due to differences in storage of the critical factor or to differences in ability to make adaptation to an unfavorable diet. In order to determine if there were any significant trends in hatchability during the course of the experiment, the hens characterized by low, intermediate, and high hatchability were divided into groups numbered 1, 2, and 3, respectively, and continued on the basal diet. After a lapse of 4 weeks all eggs were collected and settings made at weekly intervals as before. The hatchability records of the 3 newly constituted groups were pro-

jected back to the beginning of the previous experiment by combination of the records of the individuals for this period. The results, covering a period of 17 lunar months, are plotted in figure 1.

The consistency with which the 3 groups maintained their relative positions is striking. It would be difficult to explain the long-continued difference in hatchability between groups 1 and 3 on the basis of storage. Neither does adaptation to

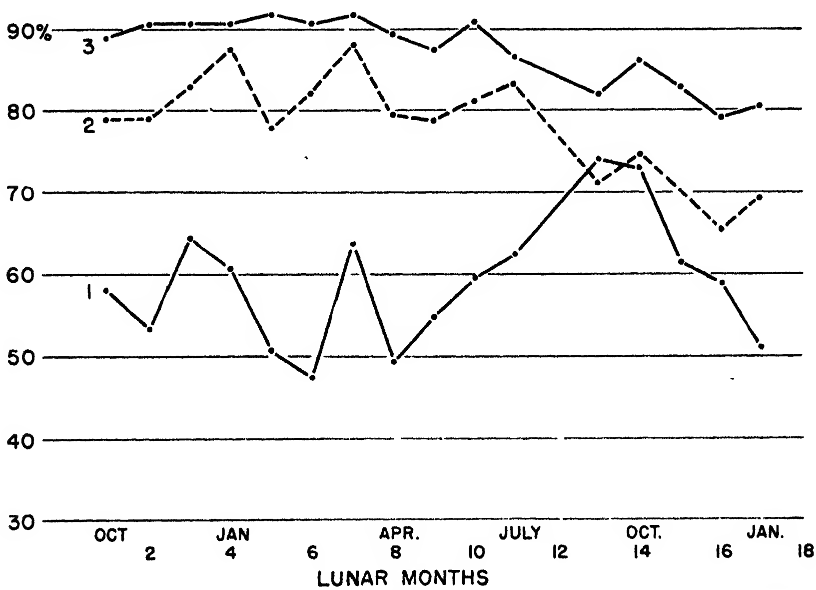


Fig. 1 Hatchability of fertile eggs of hens fed deficient diet. Groups segregated on basis of hatchability.

the diet seem a promising explanation since one would have to assume that the hens of group 3 accomplished this during the first month, those of group 2 made a partial adaptation during the same time but no subsequent improvement, and those of group 1 were incapable of making the adaptation. The sustained increase achieved by group 1 between April and September and the subsequent decline may have been seasonal although the increase came somewhat later than the usual seasonal increase.

Growth promoting effects of hen manure

Rubin et al. ('46) reported that the urine-free feces of hens were as effective as cow manure in promoting growth of chickens, even though the hens had been fed a diet that was low in the growth factor. The total excrement of hens was less effective presumably due to the presence of urinary products. Although the variation in response of the hens to the deficient diet was not related to coprophagy, it was thought that it might be due to differences in extent of synthesis of the critical factor in the digestive tracts of different hens. Accordingly the total excrement of groups 1 and 3 was collected, dried and fed to growing chickens as 5% of the basal diet during the first 6 weeks of life. Comparable groups of growing chickens were fed the same level of urine-free feces of hens, and the excrement of hens fed the positive control diet which supported hatchability at optimal or near optimal levels. These supplements replaced 5% of corn. The results are summarized in table 3. They were reported in part in the paper by Rubin et al. ('46).

TABLE 3

Average weights of chickens at 6 weeks of age as affected by feeding 5% manure.

SOURCE OF MANURE	AVERAGE WEIGHT OF CHICKENS
	gm
Hens of group 1 fed basal diet	454
Hens of group 3 fed basal diet	463
Urine-free feces of a hen fed basal diet	521
Hens fed positive control diet	491
None (basal diet without manure)	413
Least significant difference (19 : 1 odds)	63

No material difference in growth-promoting properties was demonstrated between the excrements of groups 1 and 3. In fact, neither provided a significant stimulus to growth according to the usually accepted standards of significance. A significant stimulus was provided by the urine-free feces of

a hen fed the basal diet and by the total excrement of hens fed the positive control diet.

It was thought that the 3 groups of hens might differ in efficiency of feed utilization or in rate of passage of feed through the digestive tract, but this did not prove to be the case. The efficiency indices of groups 1 to 3, respectively, determined by the method described by Bird and Whitson ('46) were 1.01, 1.03, and 1.04. Coloring the feed with 5% of charcoal did not reveal any noteworthy differences in rate of passage of material through the tract.

Variation in response of progeny as related to maternal response

After redistribution of the hens into groups 1, 2, and 3 on the basis of hatchability, one entire hatch was used in an experiment to determine whether there was any relationship between the responses of dams and progeny when both were fed the deficient diets. The progeny of groups 1, 2 and 3 numbered 96, 49 and 58, respectively. In addition 66 chicks hatched from eggs of hens fed the positive control diet were used. These chicks were wing-banded and distributed among 7 pens of a brooder house so that each of 5 pens contained an approximately equal number of chicks from each group of hens. The remaining 2 pens contained a preponderance of chicks from the hens of group 1, with a few from group 3 and the hens fed the positive control diet.

The average growth curves to 6 weeks of age and the per cent of viability during the first week are shown in figure 2. Growth and viability showed the same trend as did hatchability. Within the 6 weeks' duration of this experiment the maternal influence showed no diminution, though all of the chicks were fed the same diet throughout. The hens of group 3 were able, in spite of their deficient diet, to maintain hatchability and viability of offspring at levels that compared favorably with the performance of the hens fed the positive control diet. However, the growth rate of their progeny was

decidedly inferior to that of the chicks from hens fed the positive control diet.

Since it had proved profitable to use the offspring of hens fed the deficient diet in assaying concentrates of the growth factor prepared from cow manure, it seemed worthwhile to determine if any further advantage would be gained by using only chicks from the hens most susceptible to the deficiency.

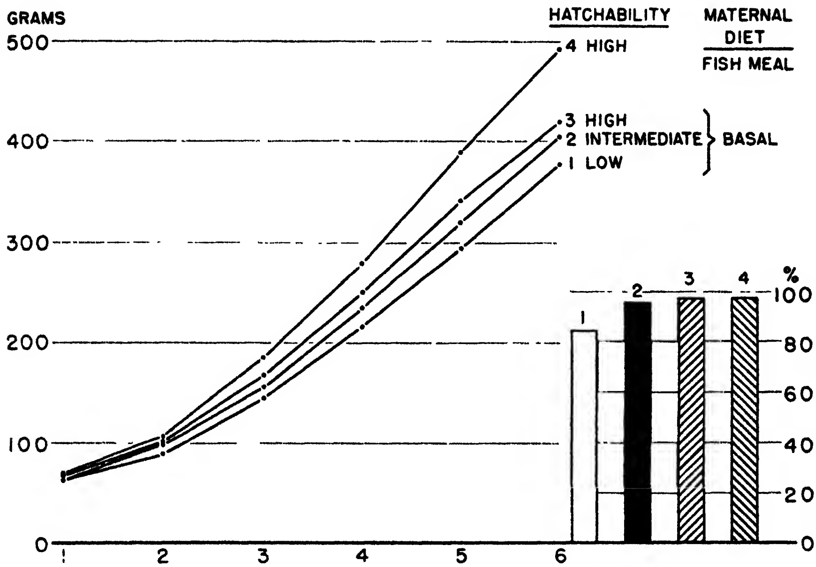


Fig. 2 Growth curves of chicks to 6 weeks of age and per cent viability (bar graphs) during first week, related to hatchability and maternal diet. Groups 1, 2, and 3 were progeny of dams fed basal diet and characterized, respectively, by low, intermediate, and high hatchability. Group 4 consisted of progeny of dams fed positive control diet containing fish meal.

It seemed possible also that such chicks might be useful in distinguishing between the effects of concentrates from manure and of other supplements. It has been reported that a growth stimulation is obtained when this diet is supplemented with calcium pantothenate and choline chloride (Bird and Rubin, '46) and when similar diets are supplemented with dl-methionine (Bird and Mattingly, '45). There is evidence,

however, that the concentrates do not owe their effect to these substances (Rubin and Bird, '46).

Two experiments were performed in which the basal chick diet and the modifications shown in table 4 were fed to chickens during the first 6 weeks of life. In experiment 1 there were in each pen 5 chicks from the hens of group 1, 12 chicks from hens of group 2, 8 chicks from those of group 3, and 8 chicks from hens fed the positive control diet. In experiment 2 each

TABLE 4

Effect of dietary supplements on growth of the progeny of deficient hens and hens naturally resistant to deficiency.

EXPERIMENT NO.	SUPPLEMENT TO BASAL DIET FOR CHICKS	AVERAGE WEIGHT OF 6-WEEK-OLD PROGENY OF: Group 1 hens (deficient) fed basal diet	Group 2 hens (intermediate) fed basal diet	Group 3 hens (resistant to deficiency) fed basal diet	Mixed hens fed positive control diet
		gm	*gm*	*gm*	*gm*
1	None	367	372	458	489
	0.05% acid ppt. of manure		511	505	532
	0.2% dl-methionine	434	451	478	541
	0.002% calcium pantothenate plus 0.1% choline chloride	393	427	501	554
2	None	331	326	472	
	0.2% dl-methionine plus 0.002% calcium pantothenate plus 0.1% choline chloride	422	405	515	

pen contained 11 chicks from the hens of group 1, 6 from group 2, and 5 from group 3. The results are shown in table 4. The figure for the progeny of the hens of group 1 fed the acid precipitate of manure extract was omitted because early mortality left too few chicks to be of any significance. The growth rates of the 3 kinds of chicks were approximately the same when all were fed the fraction prepared from manure, but this was not true when the other supplements were fed. Among the pro-

geny of the group 2 hens, the manure fraction was much superior to the other supplements, judged by growth response; among the progeny of group 3, its superiority was doubtful; and among the progeny of the hens fed the positive control diet the other supplements were at least as effective. Since, in the experiments summarized in table 4, the number of chicks fed the manure fraction was small, it may be worthwhile to point out that the results have been confirmed in numerous assay experiments in which the same fraction has elicited a maximal or near-maximal growth response in lots made up largely of the progeny of groups 1 and 2 (Rubin and Bird, '46; and unpublished data).

DISCUSSION

The results of these experiments make it clear that chickens vary widely with respect to their stores, at hatching time, of the dietary factor in question and with respect to some other characteristic which influences their ability to withstand deficiency. These 2 sources of variability combined with the variability expected in a basal diet composed of natural ingredients could account for a considerable difference in the results of supposedly duplicate experiments. In the study of the unknown factor involved here, it has proved to be profitable to use only chicks from hens fed a diet low in the factor and to eliminate the progeny of hens resistant to the deficiency, such as those of group 3. It has also proved quite essential to use in hatchability experiments only hens shown to be susceptible to the deficiency.

The variation in response to different supplements, illustrated in table 4, probably accounts for the inconsistent results of numerous experiments performed in this laboratory with chicks of uncontrolled source. In some experiments the growth response produced by methionine was as great as that produced by cow manure or by fish meal: in other experiments it was much less. It is difficult to account for the results summarized in table 4 without postulating that the unknown factor in manure, methionine and the combination of choline and

pantothenate are to some extent interchangeable for the "better" chicks. This might mean that the unknown factor is partially replaceable, and that the "better" chicks had received sufficient of it from their dams so that their remaining requirement was met by methionine or the combination of choline and pantothenic acid.

It is anticipated that future work will reveal whether or not the characteristic causing the variable response of hens to the deficient diet is inherited. This question cannot be answered on the basis of the evidence now available, since the relationship between growth response of progeny and hatchability response of dams might have been due to differences in the quantities of the essential factor stored in the eggs. There has been surprisingly little investigation of the inheritance of resistance and susceptibility to dietary deficiency. Different strains of rats have different vitamin D requirements according to Gowen ('36), different thiamine requirements according to Light and Cracas ('38), and different choline requirements according to Engel ('43), and differ with respect to efficiency of feed utilization according to Morris et al. ('33). Lamoreux and Hutt ('43) developed strains of White Leghorn chickens which varied in their requirement of riboflavin for growth. Davis et al. ('38) studied the response of 3 full sisters in each of 5 different families of White Leghorns to a diet low in riboflavin, and found that the families differed significantly with respect to hatchability of fertile eggs. However, Lamoreux ('38) reported that there was not a very close relationship between precentages of hatchability of fertile eggs produced by dams and daughters fed a diet low in riboflavin.

Whatever its basis may be, the relationship under discussion is a very close one, involving both maternal diet and maternal variability in response to diet and manifesting itself in hatchability, viability of progeny, and growth rate of progeny at least to the age of 6 weeks.

SUMMARY

Examination of the individual hatchability records of 183 hens which had been fed a diet containing no animal protein for a period of 11 lunar months revealed that 44% of the hens showed hatchability figures between 0 and 70%, 37% showed from 70 to 85% hatchability, and 19% showed from 85 to 100% hatchability. Further investigation revealed that the high hatchability maintained by the minority could not be explained on the basis of coprophagy, storage of the essential factor, or adaptation to an unfavorable diet.

Hens characterized by high and by low hatchability were not found to vary with respect to the growth promoting properties of their excreta or with respect to their efficiency of feed utilization.

The progeny of hens characterized by high, intermediate, and low hatchability, respectively, showed high, intermediate, and low viability and growth rate to 6 weeks of age. The effect of dietary supplements upon the growth of chickens varied with the maternal diets and with the ability of the dams to withstand dietary deficiency. This variation may be of considerable importance, especially in experiments in which the diets are variable because of their content of natural feedstuffs.

The variability among the hens is believed to have been inborn, as that of their progeny certainly was. Whether the variation of the progeny was congenital or hereditary cannot be stated on the basis of present evidence.

LITERATURE CITED

Bird, H. R., and J. P. Mattingly 1945 Addition of dl-methionine to starting and growing mashes. Poultry Sci., *24:* 29.

Bird, H. R., and M. Rubin 1946 Value of high levels of calcium pantothenate and pyridoxine hydrochloride in chick diets free of animal protein. Poultry Sci., *25:* 87.

Bird, H. R., M. Rubin, D. Whitson and S. K. Haynes 1946 Effectiveness of dietary supplements in increasing hatchability of eggs and viability of progeny of hens fed a diet containing a high level of soybean oil meal. Poultry Sci., *25:* 285.

BIRD, H. R., AND DONALD WHITSON 1946 Effect of diet on efficiency of egg production. Poultry Sci., *25:* 210.

DAVIS, H. J., L. C. NORRIS AND G. F. HEUSER 1938 Further evidence on the amount of vitamin G required for reproduction in poultry. Poultry Sci., *17:* 87.

ENGEL, R. W. 1943 Inherited differences in the choline requirement of rats. Proc. Soc. Exp. Biol. Med., *52:* 281.

GOWEN, J. W. 1936 Inheritance as it affects survival of rats fed a diet deficient in vitamin D. Genetics, *21:* 1.

LAMOREUX, W. F. 1938 Studies of the utilization of riboflavin by the fowl. Ph.D. Thesis, Cornell University. Reviewed by G. F. Heuser, 1938. Feedstuffs, *10:* 18.

LAMOREUX, W. F., AND F. B. HUTT 1943 Genetic resistance to deficiency of riboflavin in the chick. Genetics, *28:* 79.

LIGHT, R. F., AND L. J. CRACAS 1938 Vitamin B requirements of different strains of white rats. Science, *87:* 90.

MORRIS, H. P., T. S. PALMER AND C. KENNEDY 1933 Fundamental food requirements for the growth of the rat. VII. An experimental study of inheritance as a factor influencing food utilization in the rat. Minn. Agr. Exp. Sta. Tech. Bull. 92.

RUBIN, M., AND H. R. BIRD 1946 A chick growth factor in cow manure. II. The preparation of concentrates and the properties of the factor. J. Biol. Chem., *163:* 393.

RUBIN, M., H. R. BIRD AND I. ROTHCHILD 1946 A growth promoting factor for chicks in the feces of hens. Poultry Sci., *25:* 526.

SURFACE AREA AND METABOLISM OF GROWING GUINEA PIGS [1]

H. H. KIBLER, S. BRODY AND D. WORSTELL
Department of Dairy Husbandry, University of Missouri, Columbia

TWO FIGURES

(Received for publication November 7, 1946)

This is one of a series of reports on the metabolism of growing laboratory and farm animals, including rats, chickens (Kibler and Brody, '42; '44) cattle, horses, and swine recently summarized by Brody ('45). As in the preceding reports, surface area of the skin and body weight raised to some power (W^b) are used as reference bases.

SURFACE AREA OF THE GUINEA PIG

The guinea pig has a rather regular shape so that it appeared fairly simple to determine the surface area from appropriate linear measurements on the living animals. The following average measurements (fig. 1) were accordingly made on each animal: length and width of both sides of each ear, length and circumference of the head, length and circumference of each leg, and the length and girth of the trunk. Total surface area was then computed as the sum of the areas of these separate parts.

To check the areas computed from the linear measurements a second method was employed on the same guinea pigs. The animals were sacrificed by the use of illuminating gas, dipped in shellac and allowed to dry. Their skins were then removed

[1] Mo. Agric. Exp. Sta. Journal Series No. 1024.

and outlined on paper. The paper outlines were cut out and weighed. The skin area was then computed from the weight of the paper outline and the conversion factor for area per unit weight of paper.

The surface area data obtained by each of these 2 methods on 19 guinea pigs ranging in body weight from 230 to 1150 gm were plotted separately (fig. 1) and the power or logarithmic equation $Y = aX^b$ was fitted to each set of data by the least

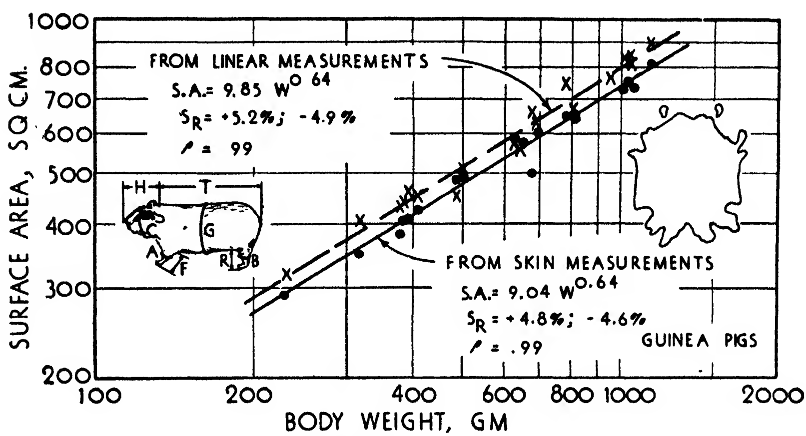


Fig. 1 Surface area of guinea pigs from linear measurements on living animals and from the measurement of the skins from the same animals. In the small figure at the left-hand side, lengths are indicated by H, head; T, trunk; F, front leg; R, hind leg. Circumferences are shown as C, head; G, trunk; A, front leg; B, hind leg. The length and width of each ear were also measured.

square method. For the linear measurements on the 19 living animals, the relation between surface area and body weight is given by the equation

$$S.A. = 9.85W^{0.64} \quad (1)$$

For the measurements of the 19 shellacked skins, the relation is expressed by the equation

$$S.A. = 9.04W^{0.64} \quad (2)$$

In both equations S.A. represents surface area in square centimeters, W represents weight in grams, and the exponent *b* indicates the relative rate of increase of surface and weight.

As b is 0.64 for both methods, the surface area increases 0.64 times as fast as the weight. The guinea pig, then, must change in form with increasing size, since — as can be shown by dimensional analysis — the exponent b would be $\frac{2}{3}$ if constant geometric similarity of form persisted during growth.

The variability of the data is about the same for both methods. The correlation coefficient[2] ρ is 0.99 for each equation (fig. 1). Two-thirds of the individual observations fall within approximately 5% of the respective equation lines as indicated by the standard errors of estimate,[3] S_R (fig. 1).

Equation 2, although parallel with equation 1, is lower presumably because of the shrinkage of the shellacked skins during drying. Therefore equation 2 was discarded and equation 1 from the linear measurement on the living animals was used for all subsequent computations.

METABOLISM

The metabolism (oxygen consumption) was measured in an eight-chamber closed circuit, volumetric apparatus (Kibler and Brody, '42). Detailed descriptions of similar multichamber volumetric appartus have been reported by Kleiber ('40), Winchester ('40), and Brody ('45, p. 325).

The measurements were made at thermoneutrality (about 30°C.). Corrections for activity were unnecessary as the guinea pigs were usually very quiet. Data were obtained during rest on each of the same 4 male and 4 female guinea pigs for the following conditions and periods: (1) non-fasting (birth to 1 year); (2) fasted for 24 hours (after 1 month of

[2] If the surface area and body weight data were perfectly correlated, the value of ρ would be 1.00. The value of 0.99 found for these data indicates a high degree of correlation that would have a probability of occurrence by chance of less than one in a hundred.

[3] $+S_R$ and $-S_R$, the standard errors of estimate, indicate the percentage range about the fitted line that includes $\frac{2}{3}$ of the data. For example, table 1 shows that a 400-gm guinea pig has a surface area of 456 cm², and figure 1 shows $+S_R = 5.2\%$ and $-S_R = 4.9\%$. The surface area derived from similar measurements on other 400-gm guinea pigs would not be expected (more than one time in three) to exceed 456 cm² by more than 23.7 cm² (0.52×456) or fall below 456 cm² by more than 22.3 cm² ($.049 \times 456$).

age to 1 year); and (3) fasted 40 to 48 hours (after 5 months of age to 1 year).

A statistical test of the covariance of metabolism and body weight failed to establish a significant difference between the oxygen consumption values on the 24- and 48-hour fasts. Therefore all fasting data were combined and designated "basal metabolism." The "resting metabolism" data, including the calorigenic action of the feed, and the "basal data" are summarized in table 1.

During growth less extensive changes occurred in the metabolic rate per unit area in the guinea pig than had been found, for example, in the rat. In the data for the guinea pig (table 1) the lowest such value is 640, occurring in the ninth month, and the highest is 846, occurring about the sixth week, a range of only 206 kilo-cal. per square meter per day. In the data for the rat (Kibler and Brody, '42) a low value of 456 was found at 1 day after birth and a high value of 1241 at 45 days of age, a range of 785 kilo-cal. per square meter per day. These differences between species are undoubtedly due to the greater physiological maturity at birth of the guinea pig than the rat.

The detailed changes in metabolism per unit of surface area with increasing age and weight are shown in table 1 for both resting and basal conditions. In general the highest values occurred between 6 and 12 weeks of age, when the body weights ranged between 200 and 400 gm. The lowest values were reached between 8 and 12 months of age at body weights between 700 and 800 gm. A striking feature of figure 2 is that the female group of guinea pigs had a consistently higher metabolic rate per unit of surface area than the male group for all ages and conditions.

The surface area base is very useful for studying age changes in metabolic rate and for equalizing metabolic rates between mature animals of different size, but metabolic rate per animal can be predicted more accurately and conveniently from the relation between total metabolism C (kilo-cal. per day) and body weight W (gm) expressed by the equation

TABLE 1

Growth and metabolism of guinea pigs.

AGE PERIOD	FEMALES: Average per guinea pig: No. observations	Body weight	Surface[1] area	Cal.[2] per day	Cal./m² per day	MALES: Average per guinea pig: No observations	Body weight	Surface[1] area	Cal[2] per day	Cal./m² per day
		gm	*cm²*				*gm*	*cm²*		
			Resting					Resting		
Week										
B–1	14	98	185	15.1	816	4	104	192	15.5	807
1–3	43	131	223	16.8	753	43	134	226	16.4	726
3–5	39	186	279	22.3	799	30	189	282	22.1	784
5–7	33	259	345	29.2	846	36	258	344	27.1	788
7–9	18	314	390	32.1	823	22	296	376	30.0	798
9–11	15	380	441	35.9	814	15	386	445	35.7	802
11–13	29	400	456	38.2	838	15	411	464	36.7	791
Month										
3–4	51	483	514	42.1	819	43	507	530	40.6	766
4–5	62	564	568	46.7	822	31	596	588	45.1	767
5–6	54	609	596	47.6	799	36	676	638	47.3	741
6–7	45	655	625	47.5	760	31	722	665	46.4	698
7–8	44	687	644	47.8	742	31	723	666	44.8	673
8–9	29	789	704	52.6	747	26	710	658	44.9	682
9–10	19	861	744	58.5	786	19	790	704	49.6	705
10–11	30	873	751	56.3	750	30	765	690	46.6	675
11–12	32	814	718	55.9	779	21	777	697	49.4	709
			Basal					Basal		
Week										
3–5	5	172	266	19.8	744	8	177	270	20.0	741
5–7	5	241	330	25.8	782	7	242	330	25.0	758
7–9	7	281	364	29.7	816	3	260	346	27.0	780
9–11	5	356	423	33.2	785	6	372	435	32.8	754
11–13	4	394	451	38.2	847	2	390	448	38.5	859
Month										
3–4	9	447	489	39.0	798	7	440	484	38.7	800
4–5	17	518	538	42.8	796	8	582	579	41.9	724
5–6	18	588	583	43.9	753	12	653	624	45.8	734
6–7	23	634	612	44.2	722	16	688	645	45.0	698
7–8	27	624	606	42.4	700	17	698	651	42.9	659
8–9	12	755	684	45.8	670	12	684	642	41.1	640
9–10	10	784	701	50.2	716	10	737	674	44.2	656
10–11	22	807	714	50.6	709	18	739	675	44.7	662
11–12	31	782	700	50.5	721	18	734	672	45.7	680

[1] Surface area was computed from equation (1) in text.

[2] The heat production was calculated on the assumption that 1 liter of oxygen has a heat equivalent of 4.9 Cal. for normally fed guinea pigs and 4.7 Cal. for guinea pigs under basal conditions.

$C = aW^b$. When this equation is fitted to data for growing animals, however, the data may have to be divided into 2 or more segments (fig. 2), since the exponent b apparently changes with growth rate (Kibler and Brody, '42).

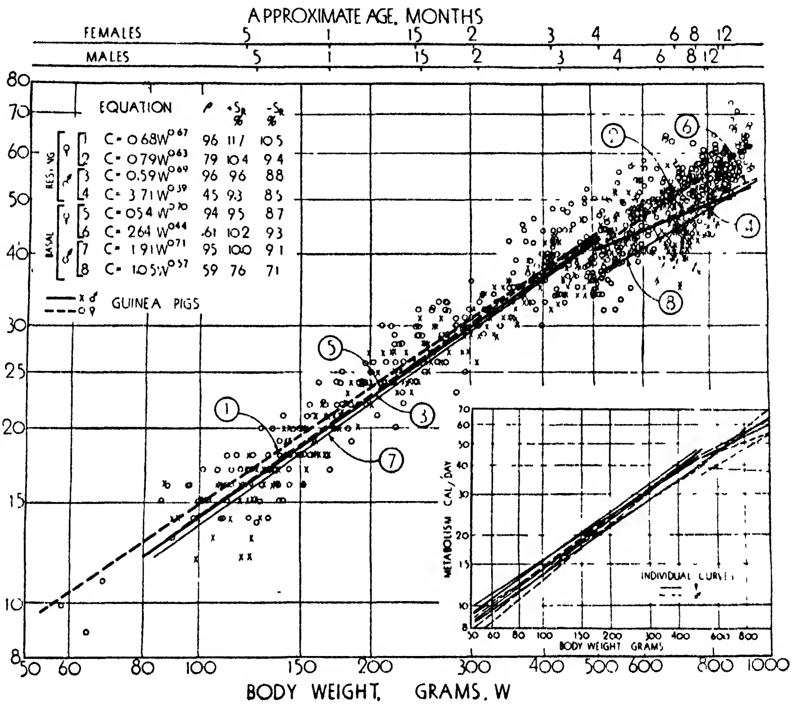


Fig. 2 Metabolism as a function of body weight in growing guinea pigs (individual observations plotted on logarithmic paper) with average age given on upper axis. Equations and statistical constants are tabulated for the male and female groups for resting and basal metabolism, but only the resting data are shown to avoid confusion. Separate curves for individual guinea pigs are shown in the inset in the lower right-hand corner.

In figure 2 the resting and basal metabolism data are plotted against body weight on logarithmically-divided paper. For the range of 50 to 500 gm body weight the equation $C = aW^b$ fits very well, the coefficients of correlation averaging 0.96 for the different curves. The 4 equation lines representing the resting and basal data for male and female guinea pigs are

nearly parallel as the values of exponent *b* vary only from 0.67 to 0.71. Above 500 gm body weight the distribution of the data is more erratic and the differences between the curves are more pronounced. The standard error of estimate, S_R, for all the metabolism data is about 10%, which is close to the values previously reported for other species. For given body weights, the fitted equation lines are higher for the female group than the male group for all data.

DISCUSSION

The lack of agreement on a reference base for equalizing metabolic rates between animals of different size is well known. It should be emphasized, however, that qualitative as well as quantitative differences appear when different bases such as body surface area, simple body weight or body weight raised to a power are used. For example, the metabolism per unit area for the guinea pig rises slightly for a few weeks after birth and thereafter declines; but the metabolism per unit weight declines from birth on. Since there is no general agreement on the reference base for energy metabolism, our results, as in the past, include data (table 1 and fig. 2) on body weight and metabolism per animal as well as metabolism in terms of unit area, and the relation between metabolism and weight raised to a power.

SUMMARY

Data are reported on metabolism (oxygen consumption) in relation to body weight, age, and surface area for the same 4 male and 4 female guinea pigs from birth to 1 year of age. The female group had a higher metabolic rate than the male group for the entire period of measurement. The average "resting" metabolism for both groups rose from 750 Cal. per square meter per day at 2 weeks after birth to 820 at 10 weeks and then declined to 720 at 7 months. Similar average "basal" values reached a maximum of 810 Cal. per square meter per day at 8 weeks and a minimum of 670 at 8.5 months.

From surface area measurements on 19 guinea pigs, ranging in weight from 230 to 1150 gm, the relation between surface area and weight was found to be expressed by the equation

$$S.A. = 9.85W^{0.64}$$

where S.A. is area in square centimeters and W is weight in grams.

We similarly related total metabolism to body weight by the same type of power equation and found that for guinea pigs weighing less than 500 gm, the metabolism increased more rapidly than surface area relative to increasing body weight (the exponent exceeded 0.64), and that for animals weighing more than 500 gm, the metabolism increased less rapidly than surface area with increasing body weight.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of June Cathey, A. J. Olsen, and Nola Miller.

LITERATURE CITED

BRODY, SAMUEL 1945 Bioenergetics and Growth. Reinhold, N. Y.

KIBLER, H. H., AND SAMUEL BRODY 1942 Metabolism and growth rate of rats. J. Nutrition, *24*: 461.

———— 1944 Metabolic changes in growing chickens. J. Nutrition, *28*: 27.

KLEIBER, MAX 1940 A respiration apparatus for serial work with small animals, particularly rats. Univ. Calif. Pub. in Physiol., *8*: 207.

WINCHESTER, C. F. 1940 Seasonal metabolic and endocrine rhythms in the domestic fowl. Mo. Agric. Exp. Sta. Res. Bull. 315.

ON THE GROWTH-PROMOTING FACTOR FOR RATS PRESENT IN SUMMER BUTTER

J. BOER, B. C. P. JANSEN AND A. KENTIE
Netherlands Institute of Nutrition and Biochemical Laboratory, University of Amsterdam, Holland

ONE FIGURE

(Received for publication August 13, 1946)

PART I — CONFIRMATION OF ORIGINAL FINDINGS

Boer and Jansen ('41) showed that summer butter contains a growth-promoting factor for rats distinct from vitamin A, vitamin D, and the essential fatty acids. The results agreed in substance with those of Schantz and his co-workers ('40) although there were certain differences between our results which will be dealt with in a later publication. Euler and his co-workers ('42, '43) reported their inability to repeat our experiments; their work will be discussed below. We have further demonstrated (Boer et al., '44) that by treating the fatty acids of summer butter with suitable adsorbing agents the growth-promoting factor can be removed from the saponifiable fraction.

Experimental

In the experiments to be described 130 male rats, 4 weeks old, were each fed 1 of 8 different diets, the basal part of all diets being, by weight, as follows: wheat flour 72 parts, casein 5 parts, yeast 10 parts, and salt mixture 3 parts. The 8 different experimental diets were made by adding to 90 gm of this mixture 10 gm of a fatty fraction which it was desired to test. The additions for successive diets were as follows:

(a) Ten per cent of the glycerol esters of the fatty acids of summer butter plus the unsaponifiable part of 2 gm of summer butter daily. The glycerol esters were prepared by heating the dried fatty acids with an equivalent quantity of anhydrous glycerol at a temperature of 150°C.

(b) Ten per cent of the glycerol esters of the fatty acids of summer butter plus 2 I.U. of vitamin D (as calciferol) and 50 μg of β-carotene daily.

(c) The same esters as under heading (a), after adsorption of the esters on fuller's earth, plus the unsaponifiable part of 2 gm of summer butter daily.

(d) The same esters as under (c) plus 2 I.U. of vitamin D (as calciferol) and 50 μg of β-carotene daily.

(e) Ten per cent peanut oil plus the unsaponifiable part of 2 gm of summer butter daily.

(f) Ten per cent of peanut oil plus 2 I.U. of vitamin D (as calciferol) and 50 μg of β-carotene daily.

(g) Ten per cent of peanut oil after adsorption on fuller's earth, plus the unsaponifiable part of 2 gm of summer butter daily.

(h) Ten per cent of peanut oil after adsorption on fuller's earth, plus 2 I.U. of vitamin D (as calciferol) and 50 μg of β-carotene daily.

The growth experiments were continued for a period of 10 weeks, with weekly weighings. We have considered especially the weights after 4 weeks and after 10 weeks, first calculating the means in such a way that only litter mates were compared, and secondly making calculations for the total number of rats on each diet. Also, we have tabulated the mean of all the rats fed diets containing summer butter esters and the mean of all the rats on diets containing peanut oil, for each of the weeks from the fourth until the tenth.

Comparison of the litter mates after the fourth week

The differences between the weights of rats on the diets containing peanut oil and those of rats on corresponding diets containing the esters of fatty acids of summer butter are

very small (see table 1). No increase in weight was noticed due to the unsaponifiable fraction of butter or to the mixture of calciferol and carotene which was administered. No difference in weight was observed between the rats receiving the glycerol esters of summer butter whether they were treated by adsorption upon fuller's earth, or were left untreated.

Comparison of litter mates after the tenth week

The difference between the effect of the summer butter esters and that of the peanut oil is evident for all comparable diets (see table 1). In 18 of the 27 pairs of litter mates the

TABLE 1

Growth of rats demonstrating the presence of a growth factor in summer butter.[1]

NUMBER OF RATS	GROUP I	GROUP II	GROUP III	GROUP IV	Groups I, II, III and IV combined.	GROUP V	GROUP VI	GROUP VII	GROUP VIII	Groups V, VI, VII and VIII combined.
	19	19	16	18		11	16	14	15	
Week										
4	99	99	97	97	98	97	89	89	87	90
5	122	123	119	120		118	111	110	109	
6	145	145	142	141	143	136	127	131	127	130
7	160	155	161	158	158	148	141	145	142	144
8	176	171	172	169	172	163	149	158	150	154
9	186	181	180	177	181	174	158	168	162	165
10	196	190	189	183	190	185	165	177	169	173

[1] Mean weights of rats by groups at the end of each week from the fourth to the tenth week of the experiment. Dietary supplements for the different groups:

Group I. Esters of the fatty acids and unsaponifiable part of summer butter.
Group II. Esters of the fatty acids of summer butter and calciferol plus carotene.
Group III. Esters after adsorption and unsaponifiable part of summer butter.
Group IV. Esters after adsorption and calciferol plus carotene.
Group V. Peanut oil and unsaponifiable part of summer butter.
Group VI. Peanut oil and calciferol plus carotene.
Group VII. Peanut oil after adsorption and unsaponifiable part of summer butter.
Group VIII. Peanut oil after adsorption and calciferol plus carotene.

growth of the rat receiving summer butter esters is better — often much better — than the growth of the litter mate receiving peanut oil. In 5 cases, the members of a pair grew equally well and in only 4 instances out of 27 was the growth better on the diet containing peanut oil. In litter mate comparison there was no significant difference between the rats receiving the unsaponifiable parts of butter and those receiving a mixture of calciferol and carotene. No significant change in the growth promoting power was obtained upon treatment of either summer butter or the peanut oil with fuller's earth as an adsorbing agent.

Comparison of whole groups after the tenth week

The rats were therefore considered as 2 groups: the first group contained all those rats which had received the summer butter acids or esters whether treated or untreated, and the second group contained all the rats which had received peanut oil whether treated by adsorption or not. The data are presented in table 1, where the body weights after 4, 5, 6, 7, 8, 9, and 10 weeks are given. After 10 weeks the mean weight of rats on the esters of the fatty acids of summer butter is 190 gm (column 6) as compared with a mean weight of rats on peanut oil of 173 gm (last column). The question arises, whether this difference of 17 gm in the mean weight of the 2 groups of rats is significant. It must first be considered, whether the sub-groups which have been combined together to make the larger groups comprise homogeneous material, statistically speaking. When the distribution of growth figures within each group is considered, there is no evidence of departure from statistically normal distribution, and therefore we are entitled to conclude that each group is statistically homogeneous. We are also entitled to compare the means of the 2 groups, and it appears that the difference of 17 gm is significant, since it is 4.6 times as great as the standard error of the difference between the 2 means. We are therefore justified in concluding that after 10 weeks there is a highly

significant difference between the weights of rats receiving summer butter acids as compared with those of rats receiving peanut oil. This difference in weight can only be attributed to the esters of summer butter.

Our data also suggest but do not establish conclusively from a statistical standpoint, that the presence of the unsaponifiable part of summer butter produces a better growth than the mixture of calciferol and carotene. A further investigation of this point is under way. It is necessary to consider now why Euler and his co-workers were not able to reproduce our results. In our opinion the explanation lies in the fact that these workers did not exactly reproduce our experimental conditions. In their experiments butter which was obtained from milk collected during March was used. According to our information, in Sweden the cows are not yet put out to pasture in the month of March. They receive at that time foodstuffs not containing fresh grass. In our observation it has been found that only summer butter (by definition butter from cows receiving fresh grass) possesses the property of promoting growth as described above. The experiments of Euler and associates were not strictly comparable with our earlier experiments, because they were not carried out with what we have defined as summer butter. A rational explanation is therefore provided to account for their inability to reproduce our experimental results.

Summary

1. Confirmation has been obtained of the growth-promoting action of the glycerol esters of the fatty acids of summer butter. An explanation has been provided of the inability of Euler and his co-workers to confirm our earlier results.

2. The treatment of summer butter with fuller's earth as an adsorbing agent does not always remove the growth-promoting factor from the glycerol esters of the summer butter acids.

3. These experiments suggest but do not conclusively establish that the unsaponifiable part of summer butter also

has a growth-promoting action apart from the action of vitamins A and D.

PART II — THE INFLUENCE OF ADSORPTION AND HYDROGENATION ON THE GROWTH-PROMOTING FACTOR OF SUMMER BUTTER

In part I we have shown that treatment of the glycerol esters of the fatty acids of summer butter with fuller's earth as an adsorbing agent did not always remove the growth-promoting factor. When adsorption treatment did produce a difference in growth, as seen earlier (Boer and Jansen, '42), this difference was never great. Further experiments have therefore been made to test the effect of adsorption upon the growth-promoting power and also to test the effect of hydrogenation of summer butter upon its growth-promoting factor.

Experimental

The summer butter was treated with an adsorbing agent in the following way: the summer butter fat was mixed with 10% by weight of dried fuller's earth, stirred for half an hour at a temperature of 80°C., and then filtered with suction through a Buchner funnel. Another portion was hydrogenated, the hydrogenation being performed at 35°C. with Raney nickel catalyst, by the method of Kentie and Nauta ('45).

A feeding experiment was then carried out with 3 groups of rats to compare the effects on growth of filtered summer butter treated with the adsorbent and of hydrogenated summer butter with that of the untreated butter. The groups contained 11 or 12 rats each, and were fed the same basal diet as was used in the experiments of Part I. Litter mates were distributed among the experimental groups as evenly as possible.

The mean results are shown in table 2. After 3 weeks on the experimental diets the untreated summer butter (see Group I) had already produced a greater growth than the filtered summer butter (13 gm — Group II). This difference

was not due to the unsaponifiable part of the butter since this part had been added to all the diets. The difference increased each week for 11 weeks of the experiment; in the last week there was a slightly greater growth due to the treated butter. In table 3 is shown the individual growth of

TABLE 2

The comparative growth promoting action of untreated summer butter (Group I), filtered summer butter after heating and stirring with fuller's earth (Group II), and hydrogenated summer butter (Group III).

WEEK OF EXPERIMENT	BODY WEIGHT IN GRAMS AT END OF WEEK		
	Group I	Group II	Group III
3	95	82	73
4	127	110	98
5			
6	165	143	124
7	178	153	127
8	192	167	146
9	203	176	158
10	211	182	169
11	220	189	174
12	231	202	187

TABLE 3

The growth of individual rats after 12 weeks of the experiment, group I given untreated summer butter, group II given filtered summer butter after heating and stirring with fuller's earth, and group III given hydrogenated summer butter. The body weights are for individual rats after 12 weeks on experiment. Rats on same line are littermates.

LITTER	GROUP I	GROUP II	GROUP III
	gm	*gm*	*gm*
1	241	175	190
1	221	195	193
2	234	199	174
3	225	209	171
7	247	207	185
7		184	204
9	242	242	202
11	241	220	185
11	207		209
12	232	197	185
13	219	192	158
Mean	231	202	187

the animals for the 12-week period. In all comparisons except one the growth on untreated summer butter was better — in many cases considerably better — than the growth on the filtrate of the same butter after treatment with fuller's earth. It may be taken that the difference between the means is real and not to be explained as due to accidental deviation of the growth of one or a few rats in the group. The difference between the means of the untreated summer butter and the filtered summer butter groups amounts to more than 3 times the standard error of these means and therefore is highly significant.

In the same way it appears from tables 2 and 3 that the growth maintained by hydrogenated summer butter is less than that which is maintained by the whole summer butter. The difference is again evident by the end of the third experimental week and increases during the following weeks until at the end of the experiment the rats receiving the summer butter have on the average gained 51 gm more than those receiving the hydrogenated summer butter. This difference is also highly significant as it amounts to 6 times the standard error of the difference between the means. Figure 1 emphasizes these differences graphically. We are therefore justified in concluding that the hydrogenation of summer butter removes its growth-promoting action. As a corollary it follows that the growth-promoting factor must be an unsaturated substance or a combination of unsaturated substances. This conclusion appears to contradict the finding of Schantz et al. ('40) that the growth-promoting factor of summer butter is a saturated compound.

An objection to the above experiments might be made, that the hydrogenated butter fat is less well absorbed from the intestinal tract because it is a hardened fat. To meet this objection we determined the fat content of the feces of the rats of the 2 groups, and found that the absorption of the normal summer butter is about 3% less than that of the hydrogenated product. As the fat-content of the diet is 10%, it follows that the rats receiving the hydrogenated summer

butter fat absorbed 0.3% less of the total diet than the rats on normal summer butter, a difference which cannot explain the great difference in growth rate.

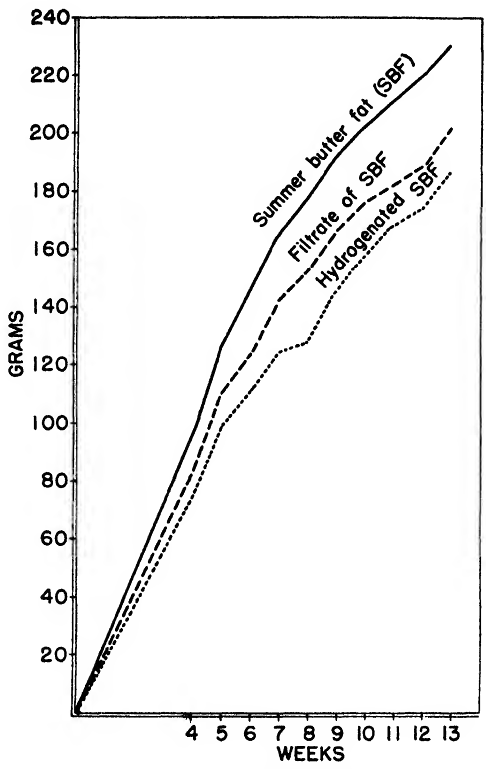


Fig. 1 The mean growth of rats receiving treated and untreated summer butter fat.

Summary

1. When summer butter fat is treated with fuller's earth the filtrate loses the growth promoting action of the original summer butter. The difference in growth is highly significant.

2. The growth produced by hydrogenated summer butter fat is quite inferior to that supported by the unchanged summer butter. This difference also is highly significant, and was shown not to be explained by different rates of absorption

of the 2 fats. Hydrogenation destroys or changes the growth-promoting factor in summer butter.

PART III — ON THE GROWTH-PROMOTING ACTION OF INDIVIDUAL SUMMER BUTTER ACIDS OBTAINED BY FRACTIONAL DISTILLATION

In our earlier experiments reported in Part I above we compared the growth of rats on summer butter with the growth when peanut oil was added to the control diet. The difference in growth between the different groups of rats appeared in 3 to 4 weeks; it was not great but increased continuously during the following weeks. The slowness with which the difference became manifest when peanut oil was used is a definite drawback which accompanies the use of this oil. In some of our earlier experiments we had obtained the impression that the growth of control rats on olive oil diminished at an earlier time than on peanut oil, so that with the shortage of peanut oil which developed during the war we changed to the use of olive oil. Later on, when this also became unobtainable, we tried rape-seed oil which gave even better results. It was found that the growth on rape-seed oil and on olive oil not only diminished at an earlier time than on peanut oil, but also, that the difference in growth between the animals receiving butter and the control rats was greater than when peanut oil was used.

The growth in the third week was already distinctly less with olive oil in the diet than when summer butter was used. After the third week the difference increased much more than when peanut oil was given instead of olive oil. For testing the various fractions prepared from summer butter the following procedure was devised. For the first 3 or 4 weeks of the experiment, all the rats received a basal diet containing olive oil (or later rape-seed oil). The litters were then divided between the 3 types of diet to be used in the latter part of the experiment, namely, the diet containing the summer butter, the diet containing the olive oil and that containing the olive oil with added fractions to be tested. It was found that

with this procedure involving the use of olive oil we were able to demonstrate a difference in growth with summer butter within 2 weeks.

Fractionation of the fatty acids of summer butter

The butter fat was saponified and the methyl esters of the fatty acids were prepared. These esters were subjected to fractional distillation at low pressures, by the method of Podbilniac, using a distillation column 2½ m in length.[1] The lower fractions up to fraction 10 (presumably C_{18}) were probably each fairly pure esters of a single acid (table 4). The higher boiling fractions were not esters of a single acid, but their fractionation served satisfactorily for a preliminary separation of active from inactive material. Each fraction was saponified and then esterified with glycerol at 150°C. (This was impossible in the case of butyric and caproic acids, whose boiling points were too low. These 2 acids were therefore esterified with ethanol.) A summary of the fractionation procedure is given in table 4.

Growth experiments with the different fractions

In the first experiment the first 4 fractions of table 4 were tested. Eighty rats, male and female, were distributed as equally as possible with regard to litter mates among 5 groups. Each group received the basal diet with an added 10% of fat. The first group received 10% olive oil. The succeeding 4 groups received fractions described under numbers 1 to 4 in table 4. In groups II, III and IV that amount of the fraction present in 20 gm of butter plus olive oil to bring the weight up to 10 gm total fat was added. The rats were maintained on the different diets for 1 week, and at the end of this time there was no difference in the growth rate of the different groups. Further experiments described below

[1] We are very much indebted to Prof. D. J. Coops who kindly permitted us to use the fractionating column in his chemical laboratory of the Free University of Amsterdam.

indicate definitely that when the growth-promoting factor is present the difference in growth between groups receiving the factor and those receiving only olive oil or rape-seed oil appears within the first week of the experiment. When there is no difference in growth found during the first week we are therefore entitled to conclude that the growth-promoting factor very probably is absent from the fraction under test.

TABLE 4

Fractional distillation at 15 mm of the methyl esters of the fatty acids of summer butter.

FRACTION NO.	WEIGHT IN GM	MOLECULAR WEIGHT	IODINE-VALUE	PRINCIPAL COMPOUNDS	DESCRIPTION
1	14		1.30	butyric acid	liquid
2	26.5	128.9	6.00	caproic acid	liquid
3	21.8	159.2	3.22	caprylic acid	liquid
4	40.6	185.7	16.36	capric acid	liquid
5	52.2	215.3	9.61	lauric acid	liquid
6	171.0	244.6	9.18	myristic acid	liquid
7	33.8	253.8	9.68	mixture	liquid
8	360.4	263.3	10.27	mixture	solid
9	93.5	279.1	35.99	mixture	half-solid
10	189.0	298.5	72.81	mixture	liquid
11a	253.4	293.4	73.44	mixture	half-solid
11b	172.3	291.6	66.80	mixture	half-solid
12	88.8	293.5	45.52	mixture	half-solid
14	24.7	277.2	73.46	mixture	solid
15	10.0	265.2	68.44	mixture	solid
16	23.3	283.3	61.44	mixture	solid
residual	54.5		38.51	mixture	solid

The conclusion is therefore justified that the growth promoting action of summer butter is not caused by the volatile fatty acids it contains, as is sometimes supposed on account of their presence in butter but not in other fats. The next 3 fractions containing chiefly lauric, myristic and tetradecenic acids, respectively, were similarly tested with negative results. In a third experiment fraction 8, fraction 9, and the heated glycerides of summer butter acids were examined with the results shown in table 5. The heating experiment

was designed to subject the natural glycerides of summer butter to the same high temperature as was used in preparing the glycerol esters after fractionation of the natural fat of the butter. The object was to test the effect of the heating upon the growth promoting factor present in the butter in order to determine whether it was stable to such a degree of heat. The growth experiment lasted 3 weeks and from table 5 it can be seen that fraction 8 contained the growth-promoting factor but that fraction 9 did not. The

TABLE 5

Growth of rats after 3 weeks on the basal diet with different added fats: group I, olive oil; group II, olive oil plus fraction 8 (table 4); group III, olive oil plus fraction 9 (table 4); group IV, heated summer butter fat.

LITTER FROM WHICH MATES WERE TAKEN	WEIGHT OF RATS			
	Group I	Group II	Group III	Group IV
	gm	*gm*	*gm*	*gm*
1	66	77	68	76
2	27	75	60	65
8	58	87	51	51
7		81	41	71
9	50	57	61	79
10	80	101	89	91
13	64	70	65	70
14	51	72	65	59
4	76	87		
6	34	65		
12	64	58		
mean	57	76	63	70

last column of the table shows that the growth on the esters exposed to a high temperature is distinctly better than the growth on olive oil. Thus we might also conclude that a high temperature does not necessarily destroy the factor wholly, although we cannot be sure that a partial destruction has not taken place. In order to extend the evidence for the existence of the growth-promoting factor present in fraction 8, we have repeated the experiment twice, first over a period of 2 weeks and then over a period of 1 week. The results

are summarized in table 6, and it is clear in each case with the shorter experiment that there is good evidence for the existence of the growth-promoting factor in this fraction.

TABLE 6

Growth of rats after 2 weeks and after 1 week on the basal ration with the addition of olive oil alone as compared with that of olive oil plus the active fraction of summer butter (group I — olive oil; group II — olive oil plus fraction 8 in table 4).

LITTER FROM WHICH MATES WERE TAKEN	WEIGHT IN GM	
	Group I	Group II
	Weight after 2 weeks on diet	
4a	38	40
6a	16	32
3a	22	42
2a	32	64
2b	33	54
3b	17	66
3b	38	45
4b	30	42
1b	10	38
mean	26	49
	Weight after 1 week on diet	
1	31	22
2	11	31
8	23	38
9	21	22
10	33	38
13	29	30
14	22	29
4	24	38
6	22	34
12	27	22
4a	21	18
6a	17	16
3a	4	22
2a	14	40
mean	21	29

Summary

1. By using olive oil or rape-seed oil and feeding all the rats in an experiment for the first 3 weeks with the olive oil diet, before dividing them into groups which are to receive the different fractions, the test for the presence of the growth-promoting factor is improved so that it can be completed in 1 or 2 weeks.

2. We have fractionated the methyl esters of the fatty acids of butter and after replacing the methyl groups by glycerol residues, we have administered the different fractions to various groups of rats in the growth tests.

3. The fractions up to C_{18} acids did not contain the growth-promoting factor, but we have succeeded in isolating a fraction beyond the C_{18} acid that did contain the growth-promoting factor.

PART IV — ISOLATION OF THE GROWTH-PROMOTING FACTOR PRESENT IN THE FATTY ACIDS OF SUMMER BUTTER

In order to account for the apparent contradiction between our results reported above, and those of Schantz et al. ('40) we have made the following working hypothesis. The growth-promoting factor is an unsaturated substance containing 18 C-atoms, which, after Twitchell separation is found in the so-called saturated fatty acid fraction. It is distinct from oleic, linoleic and linolenic acids. The only known acid which satisfies the above conditions is vaccenic acid, which has been described by Bertram ('28), and identified by him as Δ-11, 12 elaidinic acid.

Separation of vaccenic acid from the fatty acids of summer butter

The fatty acids were first separated according to the method of Twitchell ('21) and the solid unsaturated fatty acids present in the fraction designated the saturated fatty acids were isolated as described by Bertram.

Four hundred and seven gm of fatty acids derived from summer butter were dissolved in 500 ml of 96% ethanol, and to the warm solution was added a boiling solution of 130 gm of lead acetate and 1 liter of ethanol, and the mixture allowed to stand for 48 hours for crystallization of the lead soaps. The precipitated lead soaps were filtered with suction and then washed out with 250 ml of ethanol. The solid lead soaps were crystallized once more from 2 liters of ethanol, and then were boiled with hydrochloric acid (in the ratio of 1 to 1), dissolved in ether and washed out. The iodine value (Hanus) of the solid fatty acids obtained upon distillation of the ether was 8.8.

These solid fatty acids were warmed to liquefy them and added to a warm solution of 250 gm of mercuric acetate in 200 gm of acetic acid and 800 ml of 98% methanol. After standing for 24 hours the precipitated mercury salts were filtered with suction, and the filtrate cooled and at the same time 175 ml of concentrated HCl added. The acids which separated were taken up in petroleum ether, and the solution was washed with water and filtered. The petrol-ether was removed by distillation and left behind a mixture of fatty acids which was saponified. From the soap by acidification was obtained 17.1 gm of fatty acids with an iodine value of 48.9. These acids were treated once more with 50 gm of mercuric acetate, 15 ml of acetic acid and 60 ml of methanol; after saponification of the reaction mixture and regeneration of the acids we obtained 8.3 gm of solid fatty acids with an iodine value of 84.5.

In order to remove the liquid unsaturated fatty acids still present in this fraction the mass of solid acids was subjected to a Twitchell-separation ('21), following which there remained a solid fraction which weighed 2.3 gm and had an iodine value of 81.4 and a melting point of 36°. The molecular weight of the fraction was about 280, but no further investigations were carried out in order to save the small fraction for biological testing. A comparison of the above data with those of Bertram suggests that the material obtained by us is

relatively pure although it is very likely that it contains traces of saturated fatty acids and oleic acid. We obtained 0.5% yield from the butter acids, a quantity considerably greater than that obtained by Bertram (about 0.01%).

The fraction obtained in this way, consisting chiefly of vaccenic acid, was tested by growth experiments in the same way as described above. In the first experiment 29 rats were used which had received the basal diet plus rape-seed oil during a preliminary period of 4 weeks, and then were divided into 3 groups. The 3 groups then received the basal diet plus 10% summer butter, the basal diet plus 10% rape-seed oil, and the basal diet plus 10% rape-seed oil to which the vaccenic acid fraction had been added, respectively. As the exact vaccenic acid content of the summer butter was unknown

TABLE 7

First growth test comparing summer butter, rape-seed oil, and rape-seed oil with vaccenic acid.

LITTER FROM WHICH RATS WERE TAKEN	GROUP I SUMMER BUTTER Growth during successive periods			GROUP II RAPE-SEED OIL Growth during successive periods			GROUP III RAPE-SEED OIL PLUS VACCENIC ACID FRACTION Growth during successive periods		
	4 weeks on rape-seed oil	1 week on butter	18 days on butter [1]	4 weeks on rape-seed oil	1 week on rape-seed oil	18 days on rape-seed oil [1]	4 weeks on rape-seed oil	1 week on rape-seed oil fraction	18 days on rape-seed oil fraction [1]
1	53	12	50	101	33	20	94	26	41
2	77	31	36	88	22	33			
3	90	34	39	87	32	37	93	29	53
4	100	29	50	91	24	34	90	18	46
6	74	29	63	67	19	35			
7	100	30	45	88	20	32	118	26	43
7	105	30	45						
8	85	18	46	83	19	34	70	20	40
9	68	24	33	67	22	33			
10				98	24	46	82	26	44
12				77	21	34	77	19	41
12							83	22	42
11				89	22	28	72	19	38
mean	84	26	45	85	23	33	87	23	43

[1] An analysis of variance for the figures for the last 18-day period shows that the probability of these results arising from random sampling is far below 1%.

the working assumption was made that it contained twice as much as we had actually isolated in our experiment. As much vaccenic acid as will then be contained in 20 gm of butter was added to the 10 gm of rape-seed oil mixed with each 90 gm of fat-free basal diet. The results of this experiment are given in table 7 where the data on growth during the preliminary period on rape-seed oil are given, followed by those

TABLE 8

Summary of experiments confirming tests reported in table 7. Group I — summer butter; group II — rape-seed oil; group III — rape-seed oil plus the vaccenic acid fraction.

SECOND COMPARISON				THIRD COMPARISON			
Litter from which rat was taken	Growth in final 16 days with group			Litter from which rat was taken	Growth in final 16 days with group		
	I	II	III		I	II	III
	gm	gm	gm		gm	gm	gm
4	62	13	50	1	51	46	54
6	39	35	32	2	42	42	45
7	51	39	48	4	43	34	40
7		29	48	7	55	38	44
9	47	27	37	8	45	43	43
9			36	11	44	42	48
10	54	48	44	12	47	32	44
12	33	34	35	14	57	39	
14	54	32	34	14		32	
3	33	23		15		38	49
11		44	53	Mean	48	39	46
Mean	47	32	41				
Analysis of variance: probability of chance occurrence 2%.				Analysis of variance: probability of chance occurring considerably less than 1%.			

for the growth exhibited during 2 periods upon the summer butter and vaccenic acid as well as the rape-seed oil. It is the figures for the growth during the last period to which we wish to direct attention. These data were subjected to an analysis of variance by the method of Snedecor ('46), and it was found that the probability of such results occurring purely on the basis of random sampling is considerably less than 1%. We may therefore regard the results as highly sig-

nificant and ascribe the responses obtained to the dietary treatment; that is, to the addition of vaccenic acid to the basal diet as compared with the addition of rape-seed oil and summer butter.

In order to confirm this result the same experiment was repeated twice with different groups of rats, the first experiment being continued for 2 weeks and the second experiment being continued for only 1 week. The results are shown in table 8. The probability of such results being obtained on the basis of pure chance is about 2% for the second experiment and well below 1% for experiment 3. They thus confirm exactly the first experiment reported.

Summary

1. The fraction obtained from summer butter fatty acids consisting chiefly of vaccenic acid has a marked growth-promoting action. When this fraction was added to a basal diet containing rape-seed oil growth became better; the difference in growth was highly significant.

2. The growth on summer butter itself was still better than the growth on the added quantity of vaccenic acid. The difference is significant but not highly significant. No reason can be ascribed for this difference since the exact quantity of vaccenic acid in the summer butter is not known.

SUMMARY AND CONCLUSION

Earlier experiments, in which we found a better growth in young albino rats on summer butterfat than on vegetable oils, were confirmed. The growth-promoting action was mainly present in the saponifiable fraction, that is, in the fatty acids. On fractionating the methyl esters of the acids of butterfat by distillation in vacuo, the activity was found in the C_{18}-fraction. On fractionating first with lead acetate and then with mercuric acetate we obtained a fraction chiefly containing vaccenic acid. This fraction possessed distinct growth-promoting qualities. It seems highly probable therefore that vaccenic acid is a growth-promoting factor present in summer butter.

LITERATURE CITED

BERTRAM, S. H. 1928 Die Vaccensäure (Eine neue Fettsäure aus Rinder-, Schafs- und Butterfett). Biochem. Zeitschr., *197:* 433.

BOER, J., AND B. C. P. JANSEN 1941 Over de voedingswaarde van boter, vergeleken met die van andere vetten, waaraan de vitamines A en D zijn toegevoegd. Voeding Jrg., *2:* 204.

——— 1942 Investigations on the nutritive value of butter, compared with that of other fats to which varying amounts of vitamin A and D have been added. Arch. Neerland. Physiol., *26:* 1.

BOER, J., B. C. P. JANSEN, A. KENTIE AND H. K. OOSTERHUIS 1944 Proeven over de mogelijkheid van adsorptie van den groeifactor aanwezig in de vetzuren van zomerboter. Arch. Néerl. de Physiol., *28:* 57.

EULER, B. v., H. v. EULER AND I. SÄBERG 1942 Zur Kenntnis des Nährwertes verschiedener Fette. Die Ernährung Bd., *7:* 65.

——— 1943 Versuche über die Nahrungsfaktoren der Butter. Die Ernährung Bd., *8:* 257.

KENTIE, A., AND W. TH. NAUTA 1945 Hydrogenation of fatty acids in butter with Raney nickel. Rec. trav. chim., *64:* 159 (in English).

SCHANTZ, E. J., R. K. BOUTWELL, C. A. ELVEHJEM AND E. B. HART 1940 The comparative value of butterfat and certain vegetable oils. J. Dairy Sci., *23:* 181.

SNEDECOR, G. W. 1946 Statistical Methods, Ames, Iowa.

TWITCHELL, E. 1921 The precipitation of solid fatty acids with lead acetate in alcohol solution. J. Ind. and Eng. Chem., *13:* 806.

THE GROWTH-PROMOTING ACTION OF VACCENIC ACID

J. BOER, B. C. P. JANSEN, A. KENTIE AND H. W. KNOL
Netherlands Institute of Nutrition and the Laboratory for Physiological Chemistry, University of Amsterdam, Holland

(Received for publication August 13, 1946)

As demonstrated in the preceding paper, a fraction obtained from the fatty acids of summer butter and consisting chiefly of vaccenic acid had, when added to a diet with rape-seed oil, a growth promoting action similar to that of summer butter. Vaccenic acid was thought to be responsible for the growth promoting action of summer butter. However, the possibility remained that other substances were present in the fraction, and that one of these was the cause of the growth promoting action of summer butter. To prove that vaccenic acid was the active principle it was desirable to obtain it from a source other than summer butter and show that it had the same effect. Therefore, we looked for a method to obtain vaccenic acid from other material.

Böeseken, Krimpen and Blanken ('30) described a method for hydrogenation of China wood oil, a fat consisting chiefly of alpha-oleostearic acid. This acid contains 3 double bonds. By modifying the method of Boeseken and his coworkers through use of a nickel-catalyst, and by partial hydrogenation it was possible to obtain a material consisting for the greater part of vaccenic acid, an acid with a single double bond between C_{11} and C_{12}. Vaccenic acid obtained in this manner has been shown to have the same effect as the growth promoting factor from summer butter. Though we have few experimental data to report, the results appear so striking that publication of the data seems desirable.

Twenty-two rats were placed on 3 different diets, the first containing 10% summer butter, the second 10% rape-seed oil, and the third 10% rape-seed oil to which 3% hydrogenated China wood oil was added (consequently 0.3% of the total food). The basal diet was the same as described in our earlier publications. The results are given in table 1.

TABLE 1

Individual growth of rats in 4 weeks.

LITTER	SUMMER BUTTER	RAPE-SEED OIL	RAPE-SEED OIL PLUS HYDROGENATED CHINA WOOD OIL
	gm	*gm*	*gm*
1	116	66	103
3	138	79	122
4	114	74	100
7		95	103
9	107	71	90
6	112	89	
8	109	89	
2		88	114
5		78	82
Mean growth:	116	81	102

Analysis of variance shows the probability of obtaining this result by pure chance to be far less than 1%.

We feel our experiments prove that China wood oil, added to a diet with rape-seed oil, has a growth promoting action. Because the fraction of summer butter and the hydrogenated wood oil have a similar growth promoting action, and since both fractions consist chiefly of vaccenic acid, we have concluded that the growth promoting factor of summer butter is vaccenic acid.

LITERATURE CITED

Böeseken, J., J. v. Krimpen and P. L. Blanken 1930 La réduction catalytique des acides α- et β-éléostéariques sur l'influence du nickel. Rec. Trav. chim. Pays-Bas, *49:* 247.

ERRATA

The Journal of Nutrition, vol. 32, no. 6, December, 1946

Edwards, Leslie E. and others

Page 603, second line of second paragraph under *Histidine,* change 2 to 40 mg to read 2 to 40 μg.

The Journal of Nutrition, vol. 33, no. 1, January, 1947

Jukes, T. H., E. L. R. Stokstad and Margaret Belt

Page 3, line 4 from top should read —

"sodium pteroylglutamate 0.2 mg"

SOME EFFECTS OF METHIONINE ON THE UTILIZATION OF NITROGEN IN THE ADULT DOG [1]

J. B. ALLISON, J. A. ANDERSON AND R. D. SEELEY
Bureau of Biological Research, Rutgers University, New Brunswick, New Jersey

THREE FIGURES

(Received for publication June 10, 1946)

The relationship between nitrogen balance and absorbed protein nitrogen in dogs and in man is linear in the regions of negative and low positive nitrogen balances (Allison and Anderson, '45; Bricker, Mitchell and Kinsman, '45). The slope of this line is equal to the fraction of food nitrogen retained in the body of the animal and therefore, by definition, to the biological value of the dietary protein provided the excretion of body nitrogen is not affected by the dietary nitrogen. The excretion of body nitrogen, however, was shown to be a variable in protein depleted dogs fed egg white (Allison, Seeley, Brown and Anderson, '46) so that the slope of the line in these experiments was not equal to the biological value of the protein but was some function of it. The feeding of egg white nitrogen to these depleted animals conserved body nitrogen, a finding which is in agreement with data obtained in

[1] The subject matter of this paper has been undertaken in cooperation with the Quartermaster Corps Committee on Food Research.

Acknowledgment is made also for the support of the protein metabolism fund of the Bureau of Biological Research, Rutgers University.

Presented before the Division of Agricultural and Food Chemistry of the American Chemical Society, Atlantic City, April, 1946.

similar experiments on rats (Willman, Swanson, Stewart, Stevenson and Brush, '45). It has been suggested that the slope of the line be called the nitrogen balance index of the dietary protein since the slope is the rate of change of nitrogen balance with respect to nitrogen intake, a function of but not necessarily equal to the biological value (Allison, Seeley, Brown and Anderson, '46; Allison, Anderson and Seeley, '46).

Further information is needed however to evaluate this concept of food nitrogen affecting the excretion of body nitrogen. A review of the literature shows that methionine reduces the excretion of nitrogen in normal adult dogs receiving a protein-free diet (Miller, '44). Stevenson, Swanson, Willman and Brush ('46) have reported also a nitrogen sparing action of methionine in the rat. It seemed possible that this amino acid could effect a nitrogen sparing action in normal dogs similar to that found for egg white in protein depleted animals. Studies were planned therefore, to evaluate the effect of methionine on the excretion of nitrogen and on the nitrogen balance indexes of dietary proteins in normal adult dogs.

METHODS

The isocaloric diets and experimental techniques in these studies were the same as those described previously (Allison and Anderson, '45; Allison, Seeley, Brown and Anderson, '46).

The dogs were healthy normal mongrels, free from parasites. They weighed from 8 to 11 kg. The urine and feces were collected daily, 4-day collections being pooled for analysis. A preliminary feeding period of 4 days preceded the collection period in those experiments in which the nitrogen balance indexes were determined. The urine and feces were analyzed for total nitrogen by the Pregl micro-Kjeldahl method using selenium oxychloride as the catalyst. Urine urea and ammonia were determined by the aeration procedure of Van Slyke and Cullen ('16). The method of Folin ('14) was used to determine the urine creatine and creatinine.

RESULTS

Table 1 is a summary of average data obtained on 3 dogs fed consecutively a protein-free diet, a casein diet, and an egg white diet, dl-methionine being added to each diet during 1 of the 4-day collection periods. The dogs were fed the protein-free diet for 4 days (period 1) during which time the

TABLE 1

Average data obtained on 3 dogs fed dl-methionine and other sources of nitrogen. The data were obtained consecutively, each set representing the average results of a 4-day collection period. Absorbed nitrogen, urinary nitrogen, and nitrogen balance are expressed as gm/day/m² of body surface area.

PERIOD NO	NITROGEN SOURCE	ABSORBED NITROGEN GM/DAY/M²	URINE NITROGEN GM/DAY/M²	NITROGEN BALANCE GM/DAY/M²
1	protein free	0	2.56	— 3.02
2	dl-methionine [1]	0.24	1.88	— 2.27
3	protein free	0	1.82	— 2.32
4	protein free	0	2.65	— 3.18
5	casein	1.80	2.99	— 1.50
6	casein + dl-methionine [1]	2.04	1.78	— 0.22
7	casein	1.80	1.49	— 0.19
8	casein	1.80	2.33	— 1.04
9	egg white	1.96	1.79	— 0.29
10	egg white + dl-methionine [1]	2.10	1.35	+ 0.35
11	egg white	1.96	1.51	0
12	egg white	1.96	1.52	— 0.04
13	egg white	1.96	1.99	— 0.45

[1] An average of 0.24 gm of dl-methionine nitrogen was fed to each dog each day/m² of body surface. All of the amino acid nitrogen was absorbed.

average excretion of urine nitrogen was 2.56 gm and the nitrogen balance was — 3.02 gm/day/m² of body surface area. An average of 0.24 gm/day/m² of dl-methionine nitrogen was added to the protein-free diet for the next 4 days (period 2). All of the amino acid nitrogen was absorbed. The excretion of urinary nitrogen was reduced during this period to 1.88 gm, the nitrogen balance being increased to — 2.27 gm/day/m². This body nitrogen sparing action of methionine, similar to

that reported by Miller ('44), was carried over into the third period even though the amino acid had been removed from the diet. The excretion of nitrogen returned to control values during period 4.

The protein-free diet was supplemented with casein during period 5. The excretion of nitrogen in the urine increased during this period to 2.99 gm/day/m^2, an increase which would be expected from previous studies on the nitrogen balance index of casein (Allison and Anderson, '45). Addition of the same amount of methionine to this casein diet (period 6) as was previously added to the protein-free diet resulted in a marked decrease in the excretion of urinary nitrogen (to 1.78 gm/day/m^2) and an increase in the nitrogen balance almost to equilibrium (to — 0.22 gm/day/m^2). Supplementation with methionine reduced the excretion of nitrogen more when the dogs were fed the casein than when they were fed the protein-free diet. These data can be interpreted to mean that methionine is supplementing both body and casein nitrogen. The reduction in excretion of nitrogen was carried over into period 7 when the dogs received casein unsupplemented with methionine.

The excretion of urinary nitrogen and the nitrogen balance in period 8 were approaching the initial values for casein (period 5) when egg white was substituted for casein in period 9. Egg white caused a decrease in the excretion of urinary nitrogen (period 9) from that previously found for casein (period 8). This decrease in excretion of urinary nitrogen through feeding egg white agrees with previous experiments (Allison, Seeley, Brown and Anderson, '46). The addition of methionine to egg white (period 10) caused urinary nitrogen to decrease and the nitrogen balance to increase but these changes were not as marked as in the experiments on the casein diet. As in the previous experiments the sparing action was carried over into periods when the diet contained no additional methionine above that present in the egg white.

Figure 1 illustrates average urinary nitrogen excretions in 3 dogs receiving the protein-free diet for 4 days and then the

protein-free diet supplemented with methionine for 3 4-day periods (blocks with slanted lines) followed by the unsupplemented diet again. Approximately 0.24 gm of methionine nitrogen per day per square meter of body surface area was added to the protein-free diet. These data demonstrate that the urinary nitrogen excretion remains depressed when the feeding of methionine is extended from 4 days used in the experiments recorded in table 1 to 12 days. Again the nitrogen excretion remained below control levels for several days after the methionine was removed from the diet.

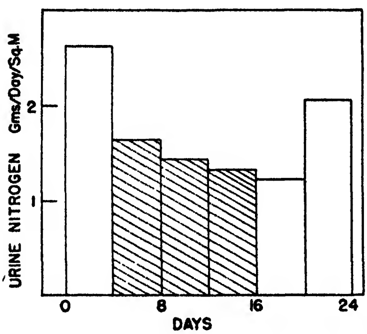


Figure 1

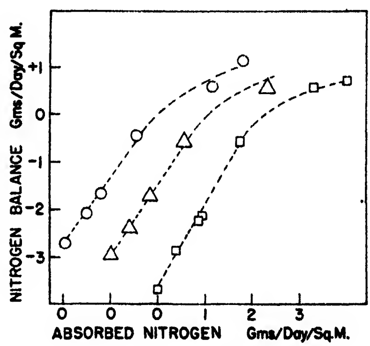


Figure 2

Fig. 1 Average daily excretion of urinary nitrogen in 3 dogs fed protein-free diet (white blocks) and protein-free diet plus 0.24 gm of dl-methionine nitrogen/day/m² body surface area (diagonal lines).

Fig. 2 Absorbed nitrogen (gm/day/m²) vs. nitrogen balance (gm/day/m²). These data were obtained while feeding 3 dogs casein diet supplemented with methionine in the ratio of 1 gm of methionine nitrogen to 14 gm of casein nitrogen.

The effect of the nitrogen sparing action of methionine on the nitrogen balance index is illustrated in figure 2 by average data obtained on 3 dogs fed casein supplemented with dl-methionine (1 gm of dl-methionine nitrogen to 14 gm of casein nitrogen). The relationship between nitrogen balance and absorbed nitrogen is linear in the region of negative nitrogen balance, becoming curvilinear in the region of positive nitrogen balance. The curvilinearity originates closer to zero nitrogen balance in methionine-supplemented than unsupplemented casein. The average slope of these lines in the

region of negative nitrogen balance is 1.5 almost double the nitrogen balance index of 0.8 for unsupplemented casein.

The nitrogen sparing action of methionine can be illustrated by applying this nitrogen balance index to the following equation derived previously (Allison, Seeley, Brown and Anderson, '46).

$$EN = NE_o - AN\ (K\text{–}BV) \qquad (1)$$

where EN is the nitrogen excreted from body sources, NE_o the excretion of nitrogen on a protein-free diet, AN the absorbed nitrogen, K the nitrogen balance index and BV the fraction of nitrogen retained in the body of the animal. Since BV can never be greater than unity the value for K of 1.5 results in a positive figure in the parenthesis of equation (1). Thus the

TABLE 2

Average nitrogen balance indexes determined on groups of 3 dogs fed the casein diet alone and the casein diet supplemented with various amounts of methionine.

$\frac{\text{METHIONINE N}}{\text{CASEIN N}}$	NITROGEN BALANCE INDEX
0.000	0.78
0.004	0.86
0.008	0.95
0.025	1.58
0.071	1.50

excretion of body nitrogen EN decreases as absorbed nitrogen (AN) increases. Feeding casein supplemented with methionine spares body nitrogen in the normal dog, therefore, even as egg white spares body nitrogen in the depleted animal. The curvilinearity of the lines in the region of positive nitrogen balance (fig. 2) proves that the sparing action decreases rapidly as nitrogen intake increases in this region. The maximum sparing of nitrogen by methionine supplemented casein is, therefore, in the region of nitrogen equilibrium.

The data in table 2 demonstrate the effect of adding different amounts of dl-methionine on the nitrogen balance index

of casein. As the ratio between methionine and casein nitrogen increases the nitrogen balance index increases gradually from approximately 0.8 for unsupplemented casein to a maximum around 1.5 for supplemented casein.

The data in figure 3 illustrate the relationship between urinary nitrogen excretion and methionine nitrogen intake, when the amino acid was added to the diet containing a constant amount of casein nitrogen (3 gm/day/m² of body surface area). These data demonstrate that as the amount of methionine nitrogen added to the casein increases the excretion of urinary nitrogen decreases rapidly to a minimum and constant value. This minimum value of excretion coincides

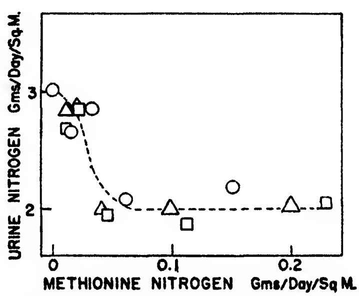


Fig. 3 Urinary nitrogen excretion (gm/day/m²) in 3 dogs fed a diet supplemented with different amounts of methionine nitrogen but containing a constant amount of casein nitrogen (3 gm/day/m² of body surface area).

with the establishment of the constant maximum nitrogen balance index of 1.5 to 1.6 recorded in table 2. There is then a definite and fixed amount of methionine necessary to produce the maximum sparing action of the casein body nitrogen mixture. Emphasis is put upon this mixture because no real separation can be made between the dietary and body nitrogen. Schoenheimer and Rittenberg ('40), for example, have demonstrated a dynamic equilibrium between tissues and the surrounding media which makes the separation of food nitrogen from body nitrogen a difficult task. The methionine is supplementing body nitrogen alone when fed with protein-free diet, but it supplements a new pattern made up of dietary

as well as body nitrogen when fed with casein or with egg white.

The data summarized in table 3 show that the decrease in the excretion of urinary nitrogen which accompanies the addition of methionine to casein is due to a decrease in the excretion of urea and not of ammonia, creatinine, or creatine. Thus methionine affects the metabolic paths involved in the synthesis of urea altering the ratio between excretion of ammonia and urea.

TABLE 3

Average data obtained on 3 dogs fed approximately 3 gm of casein nitrogen per day per square meter of body surface area. Different amounts of dl-methionine (recorded in column 1) were added to the casein. Each set of data represents the average excretion over a period of 4 days. The data were obtained consecutively with a 4-day adjustment period when the diet was changed in methionine concentration.

DL METHIONINE MG/DAY	URINARY NITROGEN			
	Creatinine mg/day/m²	Creatine mg/day/m²	Ammonia mg/day/m²	Urea mg/day/m²
0	167	35	372	1974
0	169	37	401	2054
50	157	42	407	1612
100	185	31	402	1516
0	169	36	404	1700
200	172	42	361	891
500	170	31	410	805
0	162	40	423	1646
1000	155	48	530	796

SUMMARY

1. The addition of dl-methionine to a protein-free diet or to diets containing casein or egg white will reduce the excretion of nitrogen in adult dogs. The excretion remains lower than control values for several days after the amino acid has been removed from the diet. The addition, therefore, of methionine to these diets spares nitrogen in the animal, the sparing action being more marked in the presence of casein than of egg white.

2. The relationship in normal adult dogs between nitrogen balance and absorbed casein nitrogen supplemented with

methionine is linear in the region of negative nitrogen balance and curvilinear in the region of positive nitrogen balance.

3. The slope of the line in the region of negative nitrogen balance (nitrogen balance index) increases from 0.8 in unsupplemented to from 1.5 to 1.6 in methionine supplemented casein. Slopes greater than unity prove that the addition of methionine to casein spares body and dietary nitrogen in the animal. This sparing action decreases rapidly in the region of positive nitrogen balance where the slope is a decreasing variable.

4. A definite quantity of methionine is required to produce the maximum nitrogen sparing action. The addition of 0.025 gm of methionine nitrogen to 1.0 gm of casein nitrogen produces a maximum nitrogen balance index of approximately 1.5. The addition of larger quantities of methionine to the casein does not alter this index.

5. Methionine produces the nitrogen sparing action through a reduction in the excretion of urea nitrogen, the ratio between ammonia nitrogen and urea nitrogen being increased.

ACKNOWLEDGMENT

The authors express their indebtedness to Dr. Sam Lepkovsky, Poultry Department, University of California, for his advice and encouragement during the course of this investigation.

LITERATURE CITED

ALLISON, J. B., AND J. A. ANDERSON 1945 The relation between absorbed nitrogen, nitrogen balance and biological value of proteins in adult dogs. J. Nutrition, *29:* 413.

ALLISON, J. B., J. A. ANDERSON AND R. D. SEELEY 1946 The determination of the nitrogen balance index in normal and hypoproteinemic dogs. Ann. N. Y. Acad. Sci., *57:* 245.

ALLISON, J. B., R. D. SEELEY, J. H. BROWN AND J. A. ANDERSON 1946 The evaluation of proteins in hypoproteinemic dogs. J. Nutrition, *31:* 237.

BRICKER, M., H. H. MITCHELL AND G. M. KINSMAN 1945 The protein requirements of adult human subjects in terms of the protein contained in individual foods and food combinations. J. Nutrition, *30:* 269.

FOLIN, O. 1914 On the determination of creatinine and creatine in urine. J. Biol. Chem., *17:* 469.

MILLER, L. L. 1944 The metabolism of dl-methionine and l-cystine in dogs on a very low protein diet. J. Biol. Chem., *152:* 603.

STEVENSON, G., P. P. SWANSON, W. WILLMAN AND M. BRUSH 1946 Nitrogen metabolism as influenced by level of caloric intake, character of diet and nutritional state of animal. Proc. Fed. Am. Soc. Exp. Biol., *5:* 240.

VAN SLYKE, D. D., AND G. E. CULLEN 1916 The determination of urea by the urease method. J. Biol. Chem., *24:* 117.

WILLMAN, W. P., P. P. SWANSON, G. F. STEWART, G. T. STEVENSON AND M. BRUSH 1945 Biological efficiency of egg proteins. Proc. Fed. Am. Soc. Exp. Biol., *4:* 164.

THE EFFECT OF METHIONINE UPON THE URINARY NITROGEN IN MEN AT NORMAL AND LOW LEVELS OF PROTEIN INTAKE [1]

RICHARD M. JOHNSON,[2] H. J. DEUEL, JR., MARGARET G. MOREHOUSE AND JOHN W. MEHL,

WITH THE TECHNICAL ASSISTANCE OF IRENE SMYTHE, ROBERT BABER, ROBERT BAKER, ANITA SURAN AND DALTON MERKEL

Departments of Medicine and Biochemistry, The University of Southern California School of Medicine; and the Rancho Los Amigos,[3] Los Angeles County Department of Institutions, Los Angeles

(Received for publication September 24, 1946)

Studies of the nitrogen excretion in rats and dogs on low levels of dietary nitrogen have shown that the addition of egg white protein to the diet results in a reduction of the nitrogen excretion which is not produced by casein (Swanson, '46; Allison, '45). It had previously been shown that a part of the nitrogen of certain amino acids fed singly to dogs on a low-protein diet is retained or spares body protein (Nielsen et al., '39). The same situation was found to obtain in rats (Swanson, '46), and the nitrogen-sparing effect of methionine

[1] This work was done under a contract between the University of Southern California and the Research and Development Branch, Military Planning Division of the Office of the Quartermaster General, U.S.A.

The subject matter of this paper has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

[2] Present address, Harriman-Jones Clinic, Long Beach, California.

[3] We wish to thank Mr. W. R. Harriman, Director, and Dr. Douglas W. Ritchie, Medical Director, for making the arrangements essential to this study; and to acknowledge the invaluable assistance of Miss Edmonda Hughes, Head Dietitian, in the planning and preparing of the diets.

in dogs has been confirmed (Allison, '45, '46). In these later animal experiments, the amount of body protein nitrogen which is spared is greater than the methionine nitrogen ingested. Such an effect would be of considerable importance in human nutrition under conditions of restricted food and water intake. Body protein would be conserved and less water would be required for the excretion of urea and other solutes as the result of including a relatively small amount of a single amino acid in the restricted diet.

The present studies were undertaken to determine whether such an effect is produced by methionine in men. Since it is to be expected that such an effect will most probably be observed when the caloric needs are met from non-protein sources, the bulk of the experiments were carried out at relatively high caloric intakes with protein intake of 75 gm (Diet A) or restricted to 14 gm per day (Diet B). A few experiments were also carried out at a lower caloric level, with the protein intake reduced to about 5 gm per day (Diet C). It should be recognized at the onset that the experimental periods were not prolonged, and that the subjects began each restricted period without depletion of protein stores. The conclusions can only be applied to short periods of protein restriction, and may be modified if it is possible to study subjects whose protein stores have been depleted. However, the conditions are comparable to those under which an effect has been noted in experimental animals.

EXPERIMENTAL

Subjects

The major part of the study was carried out upon 8 male volunteers who were patients at the Rancho Los Amigos. These men ranged in age from 27 to 53 years, and were not suffering from ailments which would be expected to alter their nitrogen metabolism. This expectation was borne out by the constancy of the metabolic data obtained, except for the case of subject no. 13, who showed definite evidences of metabolic disturbances during the latter part of the experiments.

The supplementary experiments at more restricted protein intakes were carried out on 4 healthy, adult, male research workers in the same age group as the other subjects.[4]

Control of the subjects. The subjects at the Rancho Los Amigos were confined to 1 ward which was used exclusively for this experiment. A nurse was in attendance at all times, and during the day 2 nurses and an attendant were on duty. Subjects were allowed to leave the ward for exercise, but only under supervision.

The normal activities of the second group of subjects were not modified during the experimental periods.

Diets

The diets were prepared in the diet kitchen of the Rancho Los Amigos, and portions were weighed onto the individual trays at the ward. The entire meal was eaten by the subjects.

Three daily menus were selected for the basal (adequate protein) series (Diet A) and 2 daily menus for the experimental (low protein) periods (Diet B). Both menus were calculated to provide 2000 Cal. The adequate protein diets were calculated to supply 12 gm of protein nitrogen per day, and were found to contain 11.1 to 12.6 gm on analysis. The low-protein diets were designed to provide 2 gm of nitrogen per day, and actually contained 2.2 to 2.3 gm nitrogen. It was not considered that an adequately palatable diet could be provided with casein as the sole source of protein, but 65% of the protein was supplied by whole milk proteins. The 2 menus for the low-protein periods are given in table 1.

In the supplementary experiments, at very low levels of protein intake, the diet consisted of 1 liter of grapefruit juice per day, containing 0.94 gm of nitrogen and supplying 400 Cal., with sucrose to supply an additional 200 Cal.; or additional sucrose and glucose candies to supply 800 Cal. (Diet C).

[4] The authors wish to thank Dr. Richard J. Winzler and Mr. Fred H. Mattson who served as subjects.

TABLE 1

Composition of the low protein diets (Diet B).

MEAL	DIET 1		DIET 2	
Breakfast	Grapefruit juice	180 gm	Orange juice	185 gm
	Cornflakes	15 gm	Rice Krispies	15 gm
	Sucrose	14 gm	Karo syrup	50 gm
	Cream, 21%	160 gm	Cream, 21%	160 gm
	Coffee	1 cup	Coffee	1 cup
	Glucose candy	11 gm	Glucose candy	16 gm
Dinner	Salad		Salad	
	Canned pear	81 gm	Lettuce leaf	38 gm
	Lettuce leaf	38 gm	Salad dressing	10 gm
	Salad dressing	10 gm	Fresh grapefruit	53 gm
	Pudding		Strained honey	15 gm
	Dry rice	20 gm	Margarine	13 gm
	Raisins	29 gm	Biscuit [1]	70 gm
	Sucrose	14 gm		
			Lemonade	
	Margarine	13 gm	Lemon juice	60 gm
	Biscuit [1]	70 gm	Sucrose	10 gm
	Coffee	1 cup		
Supper	Salad		Salad	
	Lettuce leaf	38 gm	Lettuce leaf	38 gm
	Salad dressing	10 gm	Salad dressing	10 gm
	Cobbler		Sweet potato	81 gm
	Brown sugar	27 gm	Margarine	13 gm
	Apple, peeled	100 gm		
	Biscuit [1]	70 gm	Pudding	
	Margarine	13 gm	Brown sugar	27 gm
			Tapioca	15 gm
	Whole milk	100 gm	Pineapple	94 gm
	Glucose candy	20 gm		
			Whole milk	100 gm
			Glucose candy	37 gm

[1] Biscuit recipe furnished through the courtesy of Dr. Pearl Swanson.

Corn starch	50 gm
Crisco	16 gm
Buttermilk	30 gm
Soda	¼ tsp.

Analytical methods

Nitrogen determinations were made by the Kjeldahl method. The inorganic and ethereal sulfate were determined gravimetrically, as barium sulfate, and the methionine was determined by the method of Albanese et al. ('44). Hemoglobin and plasma proteins were determined by the copper sulfate specific gravity method (Phillips et al., '43). Hemoglobin was also determined colorimetrically as acid hematin and plasma proteins by the biuret method (Mehl, '45).

Plan of the experiments

The subjects to be studied at the nitrogen intake of 2 gm were first placed on the adequate-protein diet for a period of 2 weeks. During this period and subsequently, daily 24-hour urine samples were collected, and stools were weighed but not kept for analysis. Daily records were kept of body temperature, pulse rate, respiration rate, and physical activity. The weight, blood pressure, blood count, hemoglobin, and plasma protein were obtained at weekly intervals. The nitrogen content of the diets was determined by weighing out 1 extra tray, homogenizing the food from 3 meals in a Waring blendor, and taking an aliquot for the analysis.

The preliminary period was then extended for 10 days, during which 3 gm of dl-methionine were added to the diet daily. This served to provide a somewhat longer period on Diet A, and at the same time provided information regarding the effect of methionine in subjects in nitrogen equilibrium. The methionine was then discontinued for 10 days, after which the low-protein period (Diet B) was begun. Four of the subjects were given a daily supplement of 3 gm of dl-methionine during this first low-protein period and the other 4 subjects received the low-protein diet alone. The experimental period of 10 days was followed by a recovery period of 10 days during which the subjects were returned to the adequate-protein intake, and methionine discontinued. A second 10-day experimental period was then instituted. This

provided a control period for the 4 subjects who had previously received the methionine supplement, and a methionine supplement period for the other 4 subjects. The entire experiment was concluded by a final 10-day recovery period.

The very low-protein diets (Diet C) were undertaken in a less comprehensive way, in the hope that some effect of methionine could be demonstrated at these very low levels of nitrogen intake. Laboratory workers went on the low-protein diet following their customary dietary regime, and carried on with their usual duties. One subject, no. 14, carried through 2 experimental periods of 10 days each, separated by a recovery period of 3 weeks. During the first low-protein period, a daily supplement of 3 gm of dl-methionine was provided, while the second period served as a control. Subject no. 15 received the methionine supplement during the first 7 days of a 10-day low protein period, and subject no. 16 received the methionine during the last 3 days of a 10-day period. Subject no. 17 was provided with the daily supplement of 3 gm of dl-methionine for the entire 10-day period. The data obtained on these last 4 subjects were limited to the 24-hour urinary nitrogen, inorganic and ethereal sulfate, and methionine excretions, and the body weights.

The choice of a 3-gm daily supplement of methionine is somewhat arbitrary, but was determined primarily by the levels of the methionine supplement required to give a maximum effect in rats and dogs. This amount is somewhat higher than that of 1.9 gm per day, suggested by Stare, Hegsted and McKibben ('45) as being required by a 70-kg man, though it is not sufficient to meet the requirement of approximately 4 gm postulated by Block ('43).

RESULTS

The results of the experiments employing the 2000-Cal. diet and 2 gm of nitrogen during the low-protein periods will be discussed first, since they are the most nearly complete. The serum protein, hemoglobin, and body weight have not been tabulated, since they showed no significant change during the

course of the experiments. The body weight, for example, did not vary by more than 4% during the entire period. The greatest interest attaches to the data on nitrogen and sulfur excretion. The results obtained on the basal diets are shown in table 2. Average values for the entire group are reported, and only averages for 5-day periods are recorded. Urine methionine was not determined during this period, but the urinary nitrogen, sum of ethereal and inorganic sulfate,

TABLE 2

Summary table of the effect of methionine at an adequate protein intake (Diet A).

CHEMICAL ANALYSES	PERIOD I BASAL DIET			PERIOD II BASAL DIET PLUS 3 GM METHIONINE DAILY			PERIOD III BASAL DIET	CHANGE
	Mar. 19–Mar. 23	Mar. 24–Mar. 28	Average	Mar. 29–Apr. 2	Apr 3–Apr. 8	Average	Apr 12–Apr. 15 [1]	Average of II minus I plus III
Urine N, gm/24 hr.	9.94	9.59	9.76	10.34	10.23	10.29	9.47	+ 0.63
Urine SO_3[2], gm/24 hr.	1.48	1.45	1.47	2.68	2.77	2.72	1.49	+ 1.24
Urine creatinine, gm/24 hr.	1.11	1.01	1.06	0.97	0.99	0.99	1.03	— 0.07
Food N, gm/24 hr.	12.29	11.42	11.77	12.55	11.20	11.88	11.13	+ 0.46

[1] No collections were made from April 9 to April 11, inclusive, although the subjects remained on the basal diet.

[2] Sum of inorganic and ethereal sulfate.

creatinine and food nitrogen are tabulated. The change produced by methionine is indicated in the last column of table 2, and is calculated from the difference between the averages of basal periods I and III and the basal period plus methionine, period II. There was a small increase in urinary nitrogen excretion, 0.63 gm per day, which was largely accounted for by the difference in the food nitrogen, 0.46 gm. The excess excretion of 1.24 gm SO_3 accounts for 77% of the extra sulfur ingested in the methionine, and, making allowances for the

probable excretion of sulfur in other forms, it corresponds to essentially complete metabolism of the ingested methionine.

The data obtained on the low-protein diet are summarized in table 3. Here again, averages are given for 5-day periods, except for the first basal period preceding the low-protein diet, which includes values for only 4 days. The average decrease in urinary nitrogen excretion in changing from a diet providing 11.9 gm of nitrogen to one containing 2.2 gm was 5.0 gm. The inclusion of 3 gm of dl-methionine in the low-protein period resulted in a smaller decrease in urinary nitrogen, averaging 4.7 gm per day. The smaller decrease with methionine can be accounted for by the 0.3 gm of nitrogen contained in the methionine. Likewise, the extra sulfur excretion corresponds to 82% of that ingested in the methionine, and making allowances for the fact that some additional neutral sulfur was not accounted for, this corresponds to essentially complete excretion of the methionine sulfur.

The nitrogen excretions have been recalculated in terms of the grams of nitrogen per square meter per 24 hours, and are included in table 4. These values serve to indicate the relative constancy of the results obtained with individual subjects (except for the obvious abnormalities in the case of subject no. 13), and provide some basis for comparison with the data obtained on dogs. Again, the nitrogen excretions are slightly greater with the methionine supplement. Excluding subject no. 13, the average difference was 6.1% during the first 5 days ,and 3.6% during the second 5 days on the low protein diet.

The results of the experiment with subject no. 14 are given in table 5. The two 10-day experimental periods employed a 600 Cal. diet, with a nitrogen intake of 0.94 per day. Averages of the nitrogen excretion during the last 5 days were 5.57 gm per day without methionine, and 7.12 gm per day with 3 gm of methionine daily. A total of 6.5 gm of methionine sulfur were ingested during these 5 days, and 6.3 gm more sulfur were excreted than in the control period.

TABLE 3

Summary table of the effect of methionine during a protein-low diet (Diet B).

CATEGORY OF INTEREST	METHIONINE EXPERIMENT					CONTROL EXPERIMENT					CHANGE IN URINE EXCRETION [2]	
	Basal I [1]	Low-protein-period			Basal II [1]	Basal III [1]	Low-protein-period			Basal IV[1]	Meth-ionine experi-ment	Control experi-ment
		1–5	6–10	Average 1–10			1–5	6–10	Average 1–10			
Body wt., lb.	137			136	137	137			136	136	—1	—0.5
Food nitrogen, gm	11.9 ± 0.3			2.23 ± 0.04	11.9 ± 0.3	11.9 ± 0.3			2.23 ± 0.04	11.9 ± 0.3		
Urine vol., ml	1738	1398	1373	1386	1765	1776	1349	1448	1398	1793	—366	—386
Urine nitrogen, gm	9.30	4.30	3.20	3.75	7.61	10.01	4.60	3.22	3.91	7.98	—4.71	—5.04
Urine sulfate,[3] gm SO_3	1.49	1.88	1.78	1.83	1.38	1.49	0.64	0.58	0.61	1.28	+ 0.39	—0.77
Urine methionine, gm	0.28	0.65	0.60	0.63	0.34	0.36	0.36	0.31	0.34	0.33	+ 0.32	—0.01
Urine creatinine	1.04	0.98	1.02	1.00	1.13	1.05	0.98	1.07	1.02	1.15	—0.08	—0.08

[1] Periods immediately preceding or following the low-protein diet (Diet B). All are 5 days except Basal I which was 4 days. Basal periods are those where Diet A was employed.

[2] Average of the experimental periods minus the average of the preceding and following basal period.

[3] Sum of inorganic and ethereal sulfate.

TABLE 4

Summary table of individual levels of nitrogen excretion per unit of body surface expressed as grams of nitrogen per square meter per 24 hours.

SUBJECT NUMBER	SURFACE AREA	BASAL	LOW PROTEIN		BASAL		LOW PROTEIN		BASAL	
		April 12–15	April 16–20	April 21–25	April 26–May 1	May 2–6	May 7–11	May 12–16	May 17–21	May 22–23
	m^2									
1	1.86	5.08	2.90 [1]	1.88 [1]	4.53	6.00	2.68	1.88	4.77	5.57
5	1.68	5.78	3.01 [1]	2.02 [1]	4.62	5.70	2.67	1.85	5.09	4.96
7	1.51	6.37	3.11 [1]	2.34 [1]	5.43	6.17	2.75	1.99	5.38	6.25
9	1.70	5.92	3.00 [1]	1.98 [1]	4.57	5.84	2.97	1.90	4.53	5.87
6	1.71	5.83	2.80	1.71	4.63	5.07	2.83 [1]	1.74 [1]	4.44	5.76
8	1.85	5.70	2.35	1.76	4.20	5.65	2.65 [1]	1.88 [1]	4.08	5.77
10	1.70	5.97	2.82	2.15	4.95	5.78	2.74 [1]	1.91 [1]	5.48	6.33
13	1.74	3.69	2.39	1.77	3.31	3.83	2.14 [1]	1.18 [1]	2.45	3.25

[1] Methionine periods.

The results of the other experiments employing this very low-protein intake are also recorded in table 6, where the urinary nitrogen excretions are again calculated per square meter of body surface. Urinary sulfates, as SO_3, and methionine excretions per 24 hours are also reported for subjects 15, 16, and 17. The excretion of urinary nitrogen does not reach the low values attained on the 2000-Cal. diets, but appears to respond in the same way to the addition of methionine to the diet, with a small increase.

TABLE 5

Effect of methionine on a "protein-free" diet (Diet C). Data obtained from subject no. 14 whose diet furnished 0.94 gm N and 600 Cal. per day.

DAYS ON DIET	BODY WT.		URINE N		CREATININE		SULFATE AS SO_3 [1]		METHIONINE	
	M [2]	C [3]	M	C	M	C	M	C	M	C
	lbs.	*lbs.*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
1	202	198	8.72	11.47	0.73	0.74	2.64	1.41	0.96	0.47
2	201	196	7.98	9.07	0.70	0.65	2.66	1.08	0.83	0.58
3	199	196	9.68	8.41	0.73	0.70	2.54	1.08	0.94	0.62
4	198	195	9.23	7.82	0.70	0.74	2.57	0.92	0.95	0.62
5	197	194	10.29	8.32	0.70	0.79	2.54	0.97	1.14	0.64
6	195	192	7.86	6.22	0.68	0.76	2.31	0.86	0.75	0.55
7	195	191	6.25	4.87	0.65	0.78	2.43	0.85	0.80	0.56
8	194	190	6.93	5.71	0.61	0.66	2.39	0.87	0.78	0.55
9	193	189	7.66	5.65	0.69	0.68	2.24	0.85	0.80	0.61
10	192	188	6.89	5.41	0.70	0.65	2.16	0.79	0.83	0.57

[1] Sum of ethereal and inorganic sulfate.
[2] Methionine (3 gm).
[3] Control.

DISCUSSION

The results of the experiments carried out on the 2000-Cal. diet are sufficiently consistent to conclude that the result of adding 3 gm of methionine per day had the same effect whether the nitrogen intake was 12 gm per day (Diet A), and the subjects in nitrogen balance, or whether the nitrogen intake was 2 gm per day (Diet B), and the subjects in negative nitrogen balance. In either case, the sulfur excretion increases

TABLE 6

Effect of methionine on a "protein-free" diet (Diet C).

DAYS ON DIET	URINE N (GM/M²/24 HR.)						URINE SO_4 (GM SO_3/24 HR.)			URINE METHIONINE GM/24 HR.			BODY WEIGHT (LBS.)		
	No. 14 600 Cal.	No. 14 600 Cal.	No. 14 [1] 2000 Cal.	No. 15 600 Cal.	No. 16 1200 Cal.	No. 17 1200 Cal.	No. 15 600 Cal.	No. 16 1200 Cal.	No. 17 1200 Cal.	No. 15 600 Cal.	No. 16 1200 Cal.	No. 17 1200 Cal.	No. 15	No. 16	No. 17
1	4.15 [2]	5.52	4.91	5.13 [2]	5.97	5.21 [2]	1.31 [2]	1.30	2.08 [2]	0.37 [2]	0.45	0.68 [2]	150	161	169
2	3.82 [2]	4.38	4.13	4.87 [2]	4.81	4.87 [2]	1.73 [2]	1.05	2.40 [2]	0.56 [2]	0.36	0.79 [2]	148	159	167
3	4.65 [2]	4.06	3.71	4.08 [2]	3.72	4.86 [2]	1.95 [2]	0.99	2.27 [2]	0.74 [2]	0.42	0.82 [2]	147	157	165
4	4.43 [2]	3.78	3.05	3.94 [2]	4.04	3.77 [2]	2.14 [2]	1.01	2.12 [2]	0.90 [2]	0.52	0.80 [2]	146	157	164
5	4.94 [2]	4.03	2.77	3.48 [2]	3.53	3.93 [2]	1.92 [2]	0.94	2.22 [2]	0.85 [2]	0.53	0.81 [2]	145	156	164
6	3.79 [2]	3.03	2.44	3.56 [2]	3.12	3.47 [2]	2.57 [2]	0.85	1.80 [2]	0.90 [2]	0.61	0.67 [2]	144	155	...
7	3.02 [2]	2.73	2.23			3.13 [2]			2.31 [2]		...	0.73 [2]	143	.	163
8	3.36 [2]	2.80	1.98	3.58 [2]	2.26 [2]	2.57	1.67 [2]	0.61 [2]	0.73	1.02 [2]	0.60 [2]	0.50	142	154	162
9	3.72 [2]	2.76	1.57	2.64 [2]	2.71 [2]	2.62	1.12 [2]	1.87 [2]	0.73	0.56 [2]	0.74 [2]	0.51	142	153	161
10	3.37 [2]	2.67	1.66	2.93 [2]	2.73 [2]	2.05	1.50 [2]	2.03 [2]	0.54	0.73 [2]	0.91 [2]	0.33	141	152	160
Average last 3 days	3.49 [2]	2.74	1.74	3.05 [2]	2.57 [2]	2.41									

[1] Data obtained 19 years earlier on the same subject (Deuel et al., '28).
[2] Days on which methionine was taken.

by an amount within 10% of the amount of methionine sulfur ingested and the nitrogen excretion increases slightly. The extra nitrogen, particularly in the low-protein period, can be stated to represent essentially all of the ingested methionine nitrogen. There is, thus, no indication that the added methionine spares body protein, or that its sulfur is utilized. The experiments on very low nitrogen intakes bear out this conclusion completely, and suggest a species difference from the rat and the dog.

The different results obtained with the human subject require some explanation, but must depend primarily upon an explanation of the ability of methionine or any other single amino acid to reduce the urinary nitrogen excretion on an inadequate protein intake. There are 2 similar explanations for such an effect, both of which would postulate a requirement for the amino acid in question which is not met by the breakdown of body protein and the diet. This could be a requirement for some specific function other than protein re-synthesis, or it could represent a requirement for re-synthesis of more important body proteins at the expense of less important ones. In an earlier experiment on a protein-free diet, supplying 2000 Cal., a urinary nitrogen excretion of 0.94 gm/m^2/24 hr. was reached (Deuel et al., '28). This is significantly lower than any level of nitrogen excretion reached in Allison's ('46) experiments with dogs. In either case, extra body protein would be broken down to meet this requirement, and a minimal breakdown could only be obtained when these special requirements were met by an extra dietary supplement. In the case of the human experiments, then, it would be concluded that the methionine requirement is lower, and is not a limiting factor in the attainment of minimum nitrogen excretions in these experiments, or that the requirement is met by the body protein breakdown plus any dietary protein. In this connection, it is interesting to compare the levels of urinary excretion of 1.5 to 1.9 gm N/m^2/24 hr. reached in a comparable time with dogs receiving a methionine supplement with the average levels of approximately 1.9 gm N/m^2/24 hr.

reached by these human subjects without methionine during the last 5 days of the low-protein period.

Since the addition of further methionine did not reduce the nitrogen excretion on the low-protein diet (Diet B), it can be concluded that no more methionine is required under these circumstances than that represented by the entire sulfur excretion, or 1.4 gm methionine per day for our average subject. On the average, the methionine nitrogen (calculated from the total sulfur excretion) per gm of total urinary nitrogen during the last 5 days of the low protein period at 2000 Cal. is 0.041, and this ratio is 0.035 for the last 2 days of the experiments at 600 and 1200 Cal. with subjects no. 14 and no. 17, and also on the sixth and seventh day of the low-protein experiment with subject no. 16. Since the addition of methionine did not further reduce the urinary nitrogen, this ratio is indicative of the maximum methionine requirement at these levels of nitrogen intake. The ratio obtained in a similar way for the basal periods averages about 0.035. The minimum excretion of urinary nitrogen (2 gm/m²/24 hr.) in dogs (Allison, '46) on an intake of 3 gm of casein N per m² per day was attained with a supplement of 0.04 gm of dl-methionine N per m². Since the casein furnished an additional 0.57 gm of methionine (Dunn et al., '46) or 0.053 gm of methionine-N per m² per day, the total methionine-N was at least 0.1 gm for a urinary N of 2 gm, or 0.05 gm of methionine-N per gm of urinary nitrogen. If allowance is made for the fact that the dogs were in positive nitrogen balance, the sulfur stored in protein must also be considered, and this would reduce the estimate of the ratio of methionine nitrogen to urinary nitrogen to about 0.033. Although the estimate of the ratio of methionine-N to the total urinary-N may probably be too high in the case of the men and though that for the dogs may be somewhat too low, it can be argued that these calculations indicate an appreciably higher methionine requirement in dogs than in man unless some further assumption is made regarding the fraction of the urinary sulfur which is really derived from methionine in the human experiments. It may be of some interest to note

that the ratio of urinary S to N increased during the course of the present protein-low or protein-free experiments. This increase is even more marked in the latter periods of the experiment of Deuel et al. ('28) in which the neutral sulfur excretion was relatively constant, and averaged about 0.1 gm/S/24 hr. If all of this were methionine, it would correspond to 0.46 gm/24 hr., and compares favorably with the methionine excretion of 0.6 gm obtained with the same subject in the present experiments (table 5). This excretion appears to be quite constant at different levels of protein intake (tables 3, 5, and 6), and therefore appears to be independent of the methionine intake, except insofar as the ingestion of large amounts of the amino acid itself leads to some spilling over into the urine. The ratio of sulfate-S to urinary nitrogen remains quite constant in the present experiments, but increases significantly in the longer experiment of Deuel et al. ('28) During protein deprivation, then, the body protein which is broken down becomes increasingly richer in sulfur-containing amino acids. However, the present experiments suggest that the human body is not limited in its ability to conserve nitrogen by the need to meet a methionine requirement.

The comparison of the present experiments with a 2000-Cal. intake and the 600- and 1200-Cal. intakes, as well as with the earlier experiment of Deuel et al. ('28), demonstrates the importance of an adequate calorie intake in the attainment of a minimal nitrogen excretion on a low-protein diet. The calorie intake required to establish a minimum nitrogen excretion cannot be fixed definitely from these experiments, but it can be concluded that it is greater than 1200 Cal.

SUMMARY AND CONCLUSIONS

1. Experiments have been carried out on human subjects during 10-day periods, to test the effects of added methionine on urinary excretion. Diets supplying 2000 Cal. and 75 gm of protein (Diet A), 2000 Cal. and 14 gm protein (Diet B), 1200 Cal. and 5 gm protein, or 600 Cal. and 5 gm protein (Diet C), were employed.

2. In no instance did the addition of 3 gm of dl-methionine per day to the low-protein diets decrease the urinary nitrogen excretion.

3. Under the conditions employed, methonine alone does not spare body protein, nor is there any evidence of its utilization from values for the nitrogen or sulfur excretion.

4. The nitrogen minimum ("wear and tear quota") cannot be obtained when the Caloric intake is 600 or 1200 Cal. daily although it is obtained when 2000 Cal. per day are given.

EDITORIAL NOTE

The finding reported in this paper that the requirement for methionine in man is lower than that for the dog confirms the work of Cox et al. also published in the current issue of this Journal. As a matter of fact the present paper was submitted for publication before the one by Cox et al. but its publication was delayed in an attempt to secure simultaneous publication of several papers by different authors representing work undertaken in cooperation with the Research and Development Branch, Military Planning Division of the Office of the Quartermaster General, U.S.A.

LITERATURE CITED

ALBANESE, A. A., J. A. FRANKSTON AND V. IRBY 1944 The estimation of methionine in protein hydrolysates and human urine. J. Biol. Chem., *156:* 293.

ALLISON, J. B. 1945 Effects of limited food and/or water intake Project Reports, Committee on Food Research, Military Planning Division of the Office of the Quartermaster General, Report no. 1.

——— 1946 Effects of limited food and/or water intake. Project Reports, Committee on Food Research, Military Planning Division of the Office of the Quartermaster General, Report no. 2.

BLOCK, R. J. 1943 The essential amino acid requirements of man. Yale J. Biol. Med., *15:* 723.

DEUEL, H. J. JR., I. SANDIFORD, K. SANDIFORD AND W. M. BOOTHBY 1928 The effect of 63 days of a protein-free diet on the nitrogen partition products in the urine and on the heat production. J. Biol. Chem., *76:* 391.

DUNN, M. S., M. N. CAMIEN, S. SCHANKMAN AND H. BLOCK 1946 Investigations of amino acids, peptides, and proteins XXVI. The determination of methionine in protein hydrolysates with lactobacillus fermenti. J. Biol. Chem., *163:* 577.

MEHL, J. W. 1945 The biuret reaction of proteins in the presence of ethylene glycol. J. Biol. Chem., *157:* 173.

NIELSEN, E. K., L. P. GERBER AND R. C. CORLEY 1939 The retention of the nitrogen of amino acids administered singly or in mixtures to dogs fed diets low in protein. Am. J. Physiol., *126:* 215.

PHILLIPS, R. A., D. D. VAN SLYKE, B. P. DOLE, K. EMERSON, JR., P. B. HAMILTON AND R. M. ARCHIBALD 1943 The copper sulfate method for measuring specific gravities of whole blood and plasma. U. S. Navy Bumed News Letter, *1:* 1 (no. 9).

STARE, F. J., D. M. HEGSTED AND J. M. MCKIBBEN 1945 Nutrition. Ann. Rev. Biochem., *14:* 431.

SWANSON, PEARL 1946 Utilization of nitrogen at graded levels of caloric intake. Project Reports, Committee on Food Research, Military Planning Division of the Office of the Quartermaster General, Report no. 2.

AMINO ACIDS IN NITROGEN METABOLISM WITH PARTICULAR REFERENCE TO THE ROLE OF METHIONINE [1]

MIRIAM BRUSH,[2] WANDA WILLMAN AND PEARL P. SWANSON
The Nutrition Laboratory, Foods and Nutrition Section, Iowa Agricultural Experiment Station, Ames

FOUR FIGURES

(Received for publication November 12, 1946)

Since the time of Folin ('05), the quantities of nitrogen and creatinine excreted in the urine during a period of protein deprivation have been used as a measure of "endogenous" metabolism. Data accumulated at the Iowa State College (Willman et al., '45) indicate that if the albino rat is fed a ration low in nitrogenous constituents but otherwise adequate, the nitrogen metabolism after its initial reduction proceeds at a fairly constant rate. Observations were made in 2 metabolism periods, each 7 days long, after the animals had subsisted for 11 days on the nitrogen-poor diet. However, when whole

[1] Journal paper, no. J-1404 of the Iowa Agricultural Experiment Station, Ames, project no. 799. Preliminary reports appear in the Proceedings of the Federation of American Societies for Experimental Biology, vol. 4, no. 1, 1945, and vol. 5, no. 1, 1946. The data reported are taken in large part from a thesis submitted by Miriam Brush to the Graduate College of the Iowa State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Nutrition, July, 1946. The subject matter of this paper was undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the War Department. The project has been supported in part by a grant from the Poultry and Egg National Board. This aid is gratefully acknowledged.

[2] Evaporated Milk Association fellow of the American Home Economics Association, 1945–1946.

eggs equivalent to 3.5% of protein supplemented the ration in the second metabolism period, the quantity of nitrogen eliminated in the urine was about one-third less than in the previous nitrogen-low feeding period. The excretion of creatinine was similarly altered. The depression of the so-called endogenous metabolism by the simple addition of food proteins to the basal diet casts doubt on the validity of the distinction made by Folin between endogenous and exogenous metabolism, and gives weight from another experimental approach to recent concepts of the fluidity of body processes and of the existence of a dynamic relation between food proteins and tissue proteins (Madden and Whipple, '40; Schoenheimer, '42).

The nature of the body-sparing action that follows the incorporation of eggs in the basal nitrogen-low diet of rats partially depleted of their reserve proteins has been examined in the present investigation.

The study represents a progressive series of experiments. The first investigation was planned to show whether or not the nitrogenous components were specifically responsible for the reduced excretion of nitrogen that followed the introduction of eggs into the basal ration, a mixture of the 10 essential amino acids replacing the eggs in the test diet. A suggestion of Dr. Samuel Lepkovsky [3] together with certain reports in the literature (Miller, '44; Croft and Peters, '45) that methionine may play an outstandingly important role in nitrogen metabolism led to a measurement of its influence by feeding it as the sole dietary source of nitrogen and by omitting it from the mixture of the 10 essential amino acids fed in Experiment I. The discovery that methionine was as effective as were egg proteins in depressing the urinary excretion of nitrogen formed the basis of the remaining experiments.

To determine whether or not methionine was unique in its nitrogen-sparing property, the other essential amino acids were then tested individually.

[3] Committee on Food Research, Quartermaster Food and Container Institute for the Armed Forces.

Since the quantity of nitrogen appearing in the urine of rats does not increase progressively as the quantity of egg proteins is increased (Swanson et al., '47), the response of animals to supplementary methionine offered at graded levels was investigated in the fourth experiment.

In an attempt to elucidate the nature of the physiological action of methionine, the effect of the introduction of cystine and choline into the basal ration was measured. In the last unit of the investigation, whole carcasses of animals as well as hepatic and muscular tissues were analyzed for their contents of nitrogen and methionine in an attempt to gain some information as to the utilization of nitrogen under the various experimental conditions imposed.

In the first 5 experiments, data obtained in the classical nitrogen balance test as developed by Mitchell ('24) and standardized in this laboratory (Swanson et al., '47) were used to measure the response of the experimental animals to the various dietary regimes. In the sixth experiment, the tissues examined were those of rats treated exactly like the animals in the balance experiments and sacrificed at appropriate intervals in the test.

EXPERIMENTAL PROCEDURE

The balance test

Male albino rats, approximately 6 months old, were used in the experiments. They were of Wistar stock, strain A, inbred by brother and sister matings for 94 generations. They had been reared on a stock diet which was a modification of one proposed by Steenbock in 1923 and known in this laboratory as Steenbock V (Swanson et al., '47). The various experimental groups in the first 5 experiments each contained 6 rats. Data pertaining to any rat were discarded in the final analyses only if the animal had failed to eat normally or if some acute infection had developed. This procedure was necessary only in the groups fed valine and arginine in experiment 3, the final number of rats in these groups being 2 and 4, respectively.

The effectiveness of a substance in altering the excretion of urinary nitrogen was determined by comparing the metabolic data obtained during a period when the nitrogen-low diet was fed with those secured in a subsequent interval when this basal ration was supplemented by some source of nitrogen. The test which required 29 days for completion was divided into the following periods: I. Nitrogen-low feeding period: (A) Preliminary depletion period, 11 days, (B) Collection period, 7 days; and II. Nitrogen-feeding period: (A) Adjustment period, 4 days, (B) Collection period, 7 days.

The percentage composition of the basal low-nitrogen diet containing approximately 0.6 mg of nitrogen per gm was as follows: dextrin, 73; butterfat, 10; lard, 10; Osborne and Mendel salt mixture, 4; Ruffex,[4] 2; and NaCl, 1. The ration also supplied daily, 50 mg of cod liver oil, 100 mg of rice bran polish extract, 40 μg of thiamine, 60 μg of riboflavin, 40 μg of pyridoxine, 10 mg of inositol, 10 mg of p-amino benzoic acid, 0.5 mg of nicotinic acid, 0.1 mg of calcium pantothenate, 1 mg of ascorbic acid, 5 mg of choline, 1 μg of biotin, and 0.75 mg of α-tocopherol.

The standardization of the test is described in detail elsewhere (Swanson et al., '47). Preliminary tests showed that the plane of nitrogenous metabolism had stabilized before either of the 2 collection periods was initiated. Also, points such as caloric requirements of the animals, quantity of food ingested and techniques for feeding and for collection of metabolic materials were all considered and controlled.

The animals were weighed daily in each metabolism period. The weights of the rats as recorded in table 1 represent averages of the daily weights of the animals in the 7-day interval. The data also permitted a study of the *change* in body weight that occurred from the beginning to the end of each metabolism period (table 2.) The later calculations were based on data obtained on the second and seventh days of a collection period

[4] Processed rice hulls consisting of alpha cellulose (70%), simple and hydrocelluloses; protein, fat, and vitamin-free; purchased from Fisher Scientific Co., Pittsburgh.

because the withdrawal of food necessary in shifting from 1 period of the metabolism test to another was reflected in the weight of the animal.

Evaluations have been made in terms of the relative quantities of urinary nitrogen excreted in the 2 collection periods and of "body nitrogen spared." The last index is the difference between the nitrogen balances in the 2 test periods, and represents the nitrogen that would have been lost from the body of the animal had no supplementary source of dietary nitrogen been offered in the second metabolism period.

Tissue analysis

In this phase of the experiment, 2 groups of animals were used. The first received the nitrogenous supplement at the end of the first collection period in the 29-day balance test; the second group did not. The animals were sacrificed at appropriate intervals and their tissues analyzed.

Preparation of carcasses. The animals were anesthetized with ether, after which the intestinal tract was excised and washed free of all materials. The entire carcass was then sealed in a tin container and quick-frozen at $-40°C$. Each carcass was stored at $-10°C$. until time of analysis when it was ground and transferred quantitatively to a flask containing 400 ml of 20% HCl. The mixture was autoclaved at 125°C. and 15-lb. pressure for 7 hours. These conditions assured complete hydrolysis of proteins without destruction of methionine. The solution was diluted quantitatively to volume and samples of appropriate size analyzed for nitrogen and methionine.

Preparation of hepatic and muscular tissues. The rats were starved for 10 hours prior to the time of killing. Before removal of the liver, the animals were partially bled. The liver was divided into 2 portions. About 1 gm was used for the determination of fat and moisture. The remainder was hydrolyzed in acid (4 ml of 20% HCl and 10 ml of water) at 125°C. and 15-lb. pressure.

The gastrocnemius muscles were extirpated carefully, the left was used for the determination of fat and moisture while the right was analyzed for methionine.

Analytical methods. In the estimation of methionine, aliquots of the acid digests were shaken first with small portions of activated charcoal and heated to 60°C. to remove highly colored degradation products (Lavine, '43). The filtrates were analyzed by the method described by Albanese, Frankston and Irby ('44). Interference by cystine and tryptophane was successfully controlled. Analyses were reproducible in the hands of the senior author; constant values were obtained with varying periods of autoclaving and recoveries were satisfactory (99.2%) when known quantities of crystalline methionine were added to egg proteins before autoclaving.

RESULTS

Experiment I

Egg proteins vs. the essential amino acids

In determining the role of nitrogenous constituents in the body-sparing action of dried whole eggs, 3 groups of animals were used. Group I, the negative control series, received the basal low-nitrogen diet throughout the entire experimental period of 29 days. Group II, in the second balance period, was given egg proteins equivalent to 3.5% of the quantity of ration they consumed in the first collection period. The dehydrated eggs were fed apart from the basal ration and supplied 60 mg of nitrogen daily. In the second metabolism period, Group III received 52 mg of available [5] nitrogen daily from a mixture of the 10 essential amino acids. Each acid supplied one-tenth of the total nitrogen of the mixture.

Data describing the response of the rats to the 3 dietary regimes are shown in table 1. Under the conditions of the experiment, a close relationship exists between the quantity of

[5] Those amino acids available only in the *dl-* form were incorporated in the mixture in quantities based on the relative availability of the *d* and *l* forms as reported by Sahyun ('44).

TABLE 1

Average nitrogen metabolism of rats partially depleted of bodily reserves of protein before (Period I) and after (Period II) the inclusion of a nitrogenous supplement in the basal protein-free ration.

DIETARY SUPPLEMENT IN PERIOD II	AV. BODY WT. IN PERIOD		AV CAL. EATEN IN PERIOD		URINARY N IN PERIOD		N BALANCE IN PERIOD		BODY N SPARED
	I	II	I	II	I	II	I	II	
mg nitrogen/day	*gm*	*gm*	*per day*	*per day*	*mg*	*mg*	*mg*	*mg*	*mg*
Exp. I. Egg proteins vs. the essential amino acids.[1, 2]									
Basal diet: 0	272	254	57	54	260	244	—345	—333	12
Whole eggs: 60	286	284	55	55	275	212	—384	+ 77	461
Ten Ess. A.A.: 54	240	229	46	42	319	288	—401	+ 35	436
Exp. II. Role of methionine[3] in nitrogen metabolism									
Methionine: 3	213	195	50	45	236	149	—347	—210	137
Methionine: 4	257	239	50	47	278	200	—374	—248	126
Methionine: 4	273	248	45	42	332	245	—454	—308	146
Ess. A.A.-Me: 37	260	238	46	41	308	418			
Exp. III. Methionine vs. other essential amino acids									
Methionine: 4	257	239	50	47	278	200	—374	—248	126
Arginine: 4	245	227	54	41	259	211	—353	—262	91
Isoleucine: 4	262	243	53	50	276	231	—363	—301	62
Histidine: 4	271	248	56	49	274	236	—373	—298	75
Threonine: 4	276	260	53	49	249	220	—351	—294	57
Leucine: 4	237	224	49	43	280	264	—392	—309	83
Valine: 4	280	263	59	55	307	294	—429	—390	39
Lysine: 4	248	230	52	48	259	251	—365	—301	64
Phenylalanine: 4	268	248	55	48	266	267	—371	—420	—49
Tryptophane: 4	264	244	53	47	269	288	—357	—328	29
Exp. IV. Graded doses of methionine									
Methionine: 1	262	246	52	48	334	202	—462	—293	169
Methionine: 2	255	239	47	45	332	214	—438	—270	168
Methionine: 3	213	195	50	45	236	149	—347	—210	137
Methionine: 4	273	248	45	42	332	245	—454	—308	146
Methionine: 8	261	241	48	46	317	244	—441	—285	156
Methionine: 16	270	250	57	49	332	281	—480	—266	214
Egg proteins: 15	255	241	47	45	298	216	—415	—221	194
Egg proteins: 30	256	242	48	39	309	236	—426	—135	291
Egg proteins: 43	241	233	45	43	294	216	—427	— 44	383
Egg proteins: 59	249	242	43	39	301	228	—421	— 13	408
Egg proteins: 72	261	256	45	39	292	289	—410	+ 70	480
Exp. V. Physiologically essential groups in methionine									
Methionine: 4	257	239	50	47	278	200	—374	—248	126
Cystine: 4	241	224	51	47	256	197	—338	—259	79
Choline: 4	233	217	48	45	257	221	—401	—276	125

[1] Essential amino acids abbreviated as "Ess. A.A." in column I.

[2] Methionine, isoleucine, threonine, valine, and phenylalanine were fed as the *dl*-acids. The nitrogen content of the daily dose of *dl*-threonine and *dl*-isoleucine was 8 mg.

[3] Methionine abbreviated as "Me" in column I.

nitrogen excreted when a diet poor in nitrogenous constituents is fed and the body weight of the animal (Swanson et al., '47). Therefore, the data also have been expressed graphically in terms of body surface area.

The difference in the quantity of urinary nitrogen excreted in the 2 collection periods by the negative control group probably is not significant because the mean excretion per 100 gm of body weight in each of the 2 periods of low-nitrogen feeding was 97 mg. Similar results have been obtained in 5 other experiments (Swanson et al., '47). These data show that any effects associated with the administration of the nitrogenous supplement do not reflect a concommitant change in the plane of nitrogen metabolism.

Also, other experiments were designed to permit a comparison in period II of the metabolism in the last 3 days of the adjustment period with that in the first 3 days of collection. It was found in 4 different studies that the average total nitrogen excreted (mg), respectively, in the adjustment and in the first 3 days of collection period II were: 95, 90; 117, 126; 94, 98; and 107, 108. Apparently then, the adjustment period used in the present series of experiments is long enough for the stabilization of nitrogen excretion after the nitrogenous supplement is added to the diet.

The data obtained in the present experiment, therefore, clearly show that eggs and a mixture of the 10 essential amino acids have the common property of depressing the nitrogenous excretion characteristic of the protein-free feeding period (fig. 1). The feeding of each, likewise, shifts the negative nitrogen balance of period I. When this change is expressed as "body nitrogen spared," both supplements are equally effective in their saving action on body proteins (fig. 1). The picture justifies the conclusion that the marked effects induced by the incorporation of eggs in the nitrogen-poor ration may be ascribed to their amino acid content.

It is interesting to speculate on the significance of the concept, body nitrogen spared, as used herein. It has been possible to correlate the actual loss in body weight incurred from

the second through the last day of the nitrogen-low collection period with the quantity of body tissue represented by the negative nitrogen balance in the same period. Analyses have shown that the average nitrogen content of the carcasses of 36 rats is 2.6%. This figure was used to convert the value for

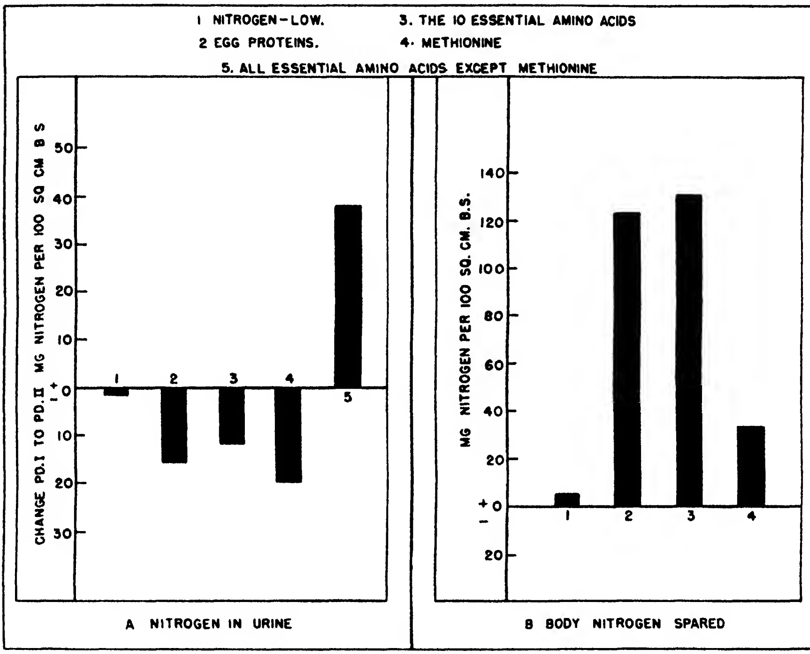


Fig. 1 Nitrogen metabolism when egg proteins, a mixture of the 10 essential amino acids, methionine, or a mixture of the 10 essential amino acids complete except for methionine supplement a basal nitrogen-low ration fed to rats.

the nitrogen balance, reduced[6] by one-seventh to correspond with the 6-day period over which the actual weight loss was observed, to its equivalent in body tissue. The observed average loss in body weight of the 26 groups of rats used in this study during the first metabolism period was 10 gm; that of the calculated value, 11 gm.

[6] A procedure necessary because food had been restricted in the first day of the 7-day collection period.

The same type of reasoning may be extended to the data obtained when the rats received a nitrogenous supplement in period II, if a basic assumption is accepted, *i.e.*, that if no dietary addition had been made, the rats would have continued to lose weight at the same rate as they had in period I. Such loss of weight occurred in the negative control group of the present experiment as well as in 4 other comparable experiments when the nitrogen-low diet was fed in both metabolism

TABLE 2

Body tissue equivalent of body nitrogen spared when some nitrogenous supplement is added to the protein-free ration of rats partially depleted of bodily reserves of protein.

ADDITION TO BASAL RATION IN PERIOD II	CHANGES IN WT. FROM 2ND TO LAST DAY OF PERIOD		BODY WT. LOSS PREVENTED BY SUPPLEMENT	BODY N SPARED (× 6¼)	TISSUE EQUIVALENT OF BODY N SPARED
	I	II			
mg nitrogen/day	*gm*	*gm*	*gm*	*mg*	*gm*
	Exp. I. Egg proteins vs. the essential amino acids				
Basal diet only: 0	— 8	—8	0	10	0
Whole eggs: 60	—11	+3	14	395	15
10 essential amino acids: 54	— 9	+6	15	374	14
	Exp. II. Role of methionine in nitrogen metabolism				
Methionine: 3	—13	—5	8	117	5
Methionine: 4	— 7	—7	0	108	4
Methionine: 4	—12	—6	6	125	5
Average	—11	—6	5	117	5

periods, the average loss in weight of 23 rats being 9 gm in period I and 8 gm in period II. On this basis then, both egg proteins and the mixture of the amino acids not only prevented the substantial weight loss (— 8 gm) that would have been induced by feeding the low-nitrogen diet but caused a definite gain in weight from the beginning to the end of the 6-day interval (table 2). Theoretically, the loss in weight *prevented* by the supplement plus the actual increment should be equivalent to the body tissue represented by *body nitrogen spared, i.e.*, the difference between the nitrogen balances in

the 2 periods. That such is the case is shown by the data presented in table 2.

Experiment II

The role of methionine in nitrogen metabolism

The effect of the addition of methionine to the basal ration was checked in 3 different experiments. Supplementary methionine equivalent to 3 mg of nitrogen was fed daily in 1 test; to 4 mg, in the other 2 tests. In each experiment, a marked and nearly identical depression in the amount of urinary nitrogen followed the introduction of the supplement (table 1). The effect was as pronounced as that induced by the addition of egg in spite of the fact that the quantities of nitrogen furnished by the 2 supplements were of different order, *i.e.*, 60 mg *vs.* 3 or 4 mg per day (fig. 1). Allison and his co-workers ('45) have also observed that the dog, if adequately depleted of its labile stores of nitrogen, responds similarly to the administration of methionine. It is important to note, however, that while methionine has some ability to spare body tissue (fig. 1), it is much less effective than either egg proteins or the mixture of amino acids. This is not a surprising observation since the metabolic requirements of the body certainly must be far too varied to permit their satisfaction by any one amino acid. The data indicate, also, that deductions as to the body sparing effect of any nitrogenous dietary material should not be made in reference to a reduced excretion of nitrogen in the urine only. This may give not only a partial, but a distorted picture.

The data depict another interesting phenomenon, *i.e.*, the specific capacity of a single substance to reduce losses of nitrogen in the urine concurrent with losses in body weight (table 2). In this instance, however, losses were reduced in magnitude. For example, the 3 groups of rats used in this experiment, on the average, lost 11 gm from the second to the last day of the first metabolism period, and 6 gm in the same interval in period II when methionine was fed. Again, on

the assumption that losses would have been of the same order in the second period as in the first had no methionine been fed, its addition to the diet prevented a loss in weight of 5 gm. Conversion of body nitrogen spared by the incorporation of methionine into the ration to its equivalent in body tissues shows that 5 gm of body weight was spared, a figure identical with the observed weight loss prevented (table 2).

The importance of dietary methionine in sparing body tissue was also demonstrated by observing the effect of its removal from the mixture of the 10 essential amino acids. Its omission caused an increase in the urinary excretion of nitrogen of 110 mg as compared with the decrease of 31 mg which followed the feeding of the complete amino acid mixture. Some of the incomplete mixture was none-the-less utilized since of the 259 mg of available nitrogen fed over the 7-day period, 149 mg did not appear as extra nitrogen in the urine. In a similar experiment, Robscheit-Robbins and Miller ('46) found that the administration of an amino acid mixture complete except for methionine caused an increase in the amount of nitrogen excreted in the urine.

Data in table 3 show that methionine behaves like egg proteins in decreasing the excretion of creatinine.

TABLE 3

Urinary excretion of creatinine when the basal low-nitrogen diet is fed (Period I) and when the same diet is supplemented with dried whole eggs or methionine (Period II).

ADDITION TO BASAL RATION IN PERIOD II	CREATININE EXCRETED IN THE 7-DAY COLLECTION PERIOD		
	Period I	Period II	Decrease in excretion
mg nitrogen/day	*mg*	*mg*	*mg*
Egg proteins: 60	106	82	24
Methionine: 4	97	70	27

Experiment III

Methionine vs. other essential amino acids

In testing the relative effectiveness of the amino acids considered essential for the nutrition of the rat, each was offered

in a dose that supplied 4 mg of available nitrogen per day, a quantity suggested by the work of Wolf and Corley ('39).

Data presented in table 1 and figure 2 show that methionine was outstanding in its ability to decrease urinary losses of nitrogen. The other amino acids depressed its excretion in varying degree.

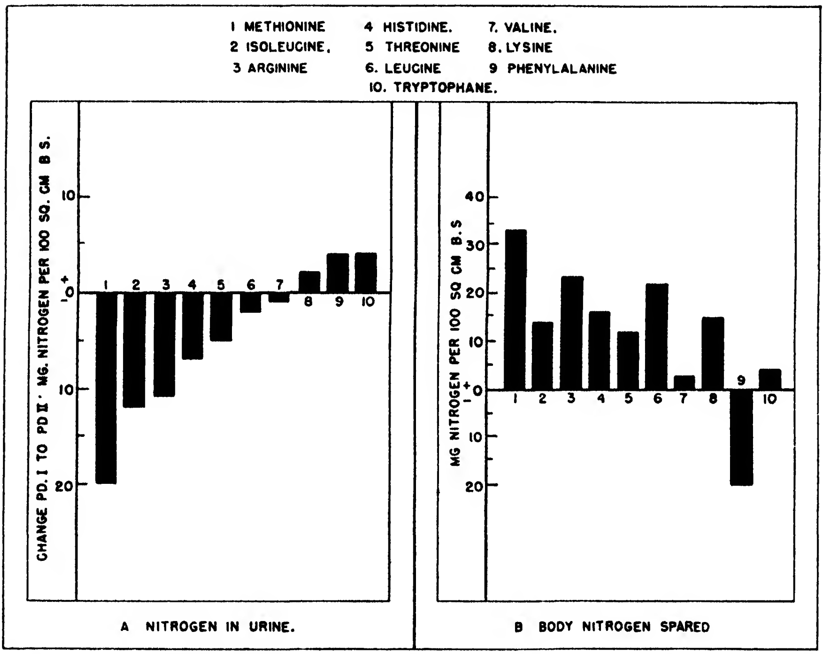


Fig. 2 Nitrogen metabolism when each of the 10 essential amino acids supplements a basal nitrogen-low ration fed to rats.

The data are even more interesting when translated in terms of body-sparing action (fig. 2). Why phenylalanine, valine, and tryptophane are the least effective members of the group is difficult to explain. Indeed, phenylalanine seems to stimulate the breakdown of body tissue, a finding that suggests that the feeding of the acid may lead to the synthesis of the related amino acid, di-iodotyrosine. Unavoidable reduction of the number of animals in the valine group to 2 rats may be reflected in the results obtained from feeding this acid.

The appearance of a bright yellow pigment in the urine of rats fed tryptophane is further evidence of the difficulty the animals experienced in utilizing this amino acid under the conditions of the experiment. The substance excreted, in this instance, was not xanthurenic acid, a compound that may appear in the urines of pyridoxine-deficient rats fed tryptophane (Lepkovsky, Roboz and Haagen-Smit, '45).

It is noteworthy that the sum of the quantities of body nitrogen spared by each amino acid when fed singly was 476 mg while the 10 amino acids fed as a mixture spared 463 mg (table 1). From this it seems that the various amino acids have specific roles in protein metabolism which they fulfill whether fed singly or as part of a mixture.

Differences in the results obtained in this study and those in a similar experiment reported by Burroughs, Burroughs and Mitchell in 1940 may be explained, it seems to the present authors, in terms of relative protein stores, the constancy of the plane of nitrogen metabolism in the period of low-protein feeding, the lag or hangover in the effect of proteins or amino acids on the excretion of nitrogen as observed in rats and dogs in subsequent periods of nitrogen deprivation (Swanson et al., '47; Allison, Anderson and Seeley, '45) and of the repeated use of the same animals without realimentation in successive experiments.

Experiment IV

Effect of feeding graded doses of supplementary methionine

Unlike the progressive increases in the quantity of nitrogen excreted in the urine that accompany graded increases in the quantity of nitrogen fed when many proteins are added to the basal diet, egg proteins maintain the depression at about the same point no matter whether 1, 2, 3, or 4% of egg proteins are furnished in the ration (fig. 3). Only when the diet contains dried eggs equivalent to 5% of protein does the excretion of nitrogen equal that in period I.

Data showing the effect of methionine when fed in quantities that supplied nitrogen ranging from 1 to 16 mg daily are presented in table 1 and may be compared with that of eggs in figure 3. Care should be exercised in comparing the graphic presentations since weight of methionine nitrogen in 1 case, and per cent of egg proteins in the other, were used as the bases for pictorial arrangement. Trends, however, are clear.

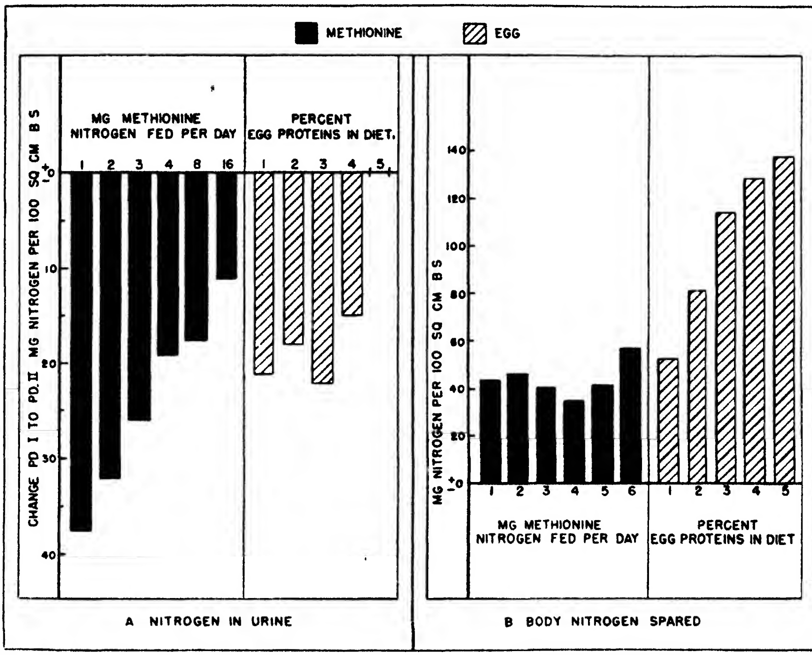


Fig. 3 Nitrogen metabolism when increasing quantities of either methionine or egg proteins supplement a basal nitrogen-low ration fed to rats.

When methionine was offered at the 6 ascending levels of dietary intake, there was a progressive decrease in the depression in the excretion of urinary nitrogen that ranged from 35 to 11 mg per 100 cm² of body surface. The data suggest that, under these experimental conditions, the methionine requirement of a rat weighing about 260 gm is 11 mg or less per day. If any excess beyond the amount required is excreted,

the urinary excretions of nitrogen characteristic of the higher levels of intake may be reasonably explained.

Increased quantities of egg proteins in the diet were associated with an increased sparing of body nitrogen (fig. 3). On the other hand, no real difference existed in the respective quantities of body nitrogen spared at each level of methionine intake as shown by analysis of variance. If the requirement is of the low order postulated, it is logical that, however large the quantities of methionine ingested, no additional body nitrogen would be spared, once the requirement is satisfied. Egg proteins, on the other hand, contain substances in addition to methionine, and as the amount of egg ingested is increased, amino acids are provided that also are needed for maintenance or for the synthesis of functional metabolites.

Experiment V

Physiologically essential groups in methionine

Is methionine important by virtue of its organic sulphur, its methyl group or both? In a comparative test, sulphur in the form of cystine and potential methyl group in the form of choline were fed as dietary supplements in quantities that were equivalent to 4 mg of nitrogen. The quantity of choline given was in addition to the amount routinely supplied in the vitamin mixture.

Data presented in table 1 and figure 4 show that cystine and choline apparently share with methionine the ability to decrease urinary losses of nitrogen and to spare body tissue. Any apparent differences are statistically insignificant. This finding agrees with the work of Mulford and Griffith ('42) which indicates that metabolically the same molecule of methionine cannot be used as a source of both organic sulphur and labile methyl groups. Were the reverse true, one molecule of methionine should be able to spare body nitrogen equivalent to the sum of that spared by cystine and choline individually.

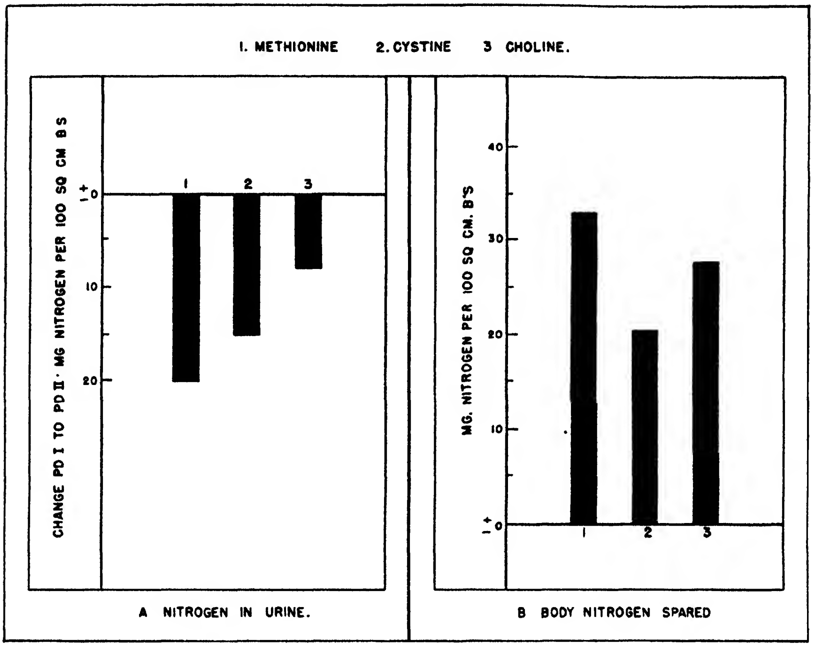


Fig. 4 Nitrogen metabolism when methionine, cystine, or choline supplements the basal nitrogen-low ration fed to rats.

Experiment VI

Tissue analyses

The carcasses of a group of animals removed from the stock colony when 6 months old, of groups fed the ration poor in nitrogenous materials for either 18 or 29 days, respectively, and of another group maintained first on the basal diet for 18 days and then on a nitrogen-supplemented diet for 11 days were analyzed for nitrogen and methionine. Two nitrogenous supplements were fed, *i.e.*, egg equivalent to 42 mg of nitrogen and methionine equivalent to either 1, 2, 4, or 16 mg of nitrogen per day. Data obtained from these groups permitted comparison of total carcass nitrogen at the beginning of the balance test, at the end of period I in the experiment, at the end of the second metabolism period in which a nitrogenous

supplement was ingested, and at the end of the second metabolism period when no supplement had been offered.

Data pertaining to these experiments are presented in table 4. It may be seen that the nitrogen content of the whole carcasses decreased from 8.38 gm to 6.41 gm as the result of feeding the nitrogen-low diet for 18 days. The body weight declined correspondingly. Neither the feeding of the basal diet for an additional 11 days nor the administration of supplementary methionine for the same period had any further effect on the nitrogen content of the carcass. The addition

TABLE 4

Average nitrogen and methionine present in carcasses of rats at different intervals of the balance test following the feeding of either a nitrogen-poor or a nitrogen-supplemented ration in period II.

OBSERVATIONS (3 RATS PER GROUP)	AT BEGINNING OF TEST PERIOD	AFTER N-LOW FEEDING FOR		AFTER 11 DAYS SUPPLEMENTAL FEEDING FOLLOWING 18 DAYS ON N-LOW DIET				
		18 days	29 days	42 mg egg N per day	Mg Me N per day			
					1	2	4	16
Av. body wt., gm	326	253	249	289 [1]	233	226	235	225
Total N, gm	8.38	6.41	6.69	7.75	6.67	6.25	5.52	6.37
N, gm %	2.58	2.52	2.68	2.69	2.86	2.77	2.36	2.82
Total Me, mg	182	132	132	159	144	123	123	145
Me, mg %	56	52	53	55	62	54	51	63
$\frac{\text{Methionine N}}{\text{Total N}}$, %	2.2	2.1	2.0	2.1	2.2	2.0	2.2	2.2

[1] The rats in this group were large, weighing 296 gm after the feeding of a low-nitrogen ration for 18 days.

of egg proteins to the diet for 11 days, however, brought the nitrogen content of the carcass to 7.75 gm.

The total methionine content of the carcass fell during the first 18 days that the nitrogen-low diet was fed, and the data seem to indicate that no further changes were induced thereafter, regardless of whether the animal was continued on the ration poor in nitrogen or whether it was given the basal diet supplemented with methionine. Apparently the body sparing action of methionine (fig. 4) is not related to a gain in either the total nitrogen or the methionine content of the

carcass. This observation is in accord with the relatively small amount seemingly required by the adult rat.

The findings suggest that methionine either serves in the fabrication of essential metabolites contributing little nitrogen to the total value of the carcass or acts as a catalytic agent governing essential processes. After the basal need is met, the methionine may then be used in the regeneration of body tissues providing the other building stones are present, as indicated by the fact that the methionine content of whole carcasses of rats fed egg proteins is higher than that of those fed the basal diet alone or the basal ration supplemented with methionine. The ratio of methionine nitrogen of

TABLE 5

Average weight, total nitrogen content, and methionine content of livers extirpated from rats at end of each period of the balance test, 43 mg of methionine supplementing the ration in period II.

NO. OF RATS	BODY WT.	WT. OF FRESH LIVER	WT. OF DRY FAT-FREE LIVER	TOTAL NITROGEN IN LIVER	NITROGEN IN DRY FAT-FREE LIVER	TOTAL METHIONINE IN LIVER	METHIONINE N / TOTAL N
	gm	*gm*	*gm*	*mg*	*%*	*mg*	*%*
N-low diet for 18 days							
4	267	6.41	1.50	127	8.46	41	3.00
N-low diet for 18 days followed by methionine supplementation for 11 days							
5	290	6.96	1.47	154	10.4	50	2.94

the carcass to its total nitrogen content remained unchanged, *i.e.*, 2.1, in spite of any dietary manipulation imposed.

Methionine feeding for the last 11 days of the metabolism test apparently is associated with the elaboration of some hepatic tissue. Average weights of the dry fat-free livers of the animals killed at the end of each experimental period were approximately the same. However, the livers of the animals fed the nitrogen-low diet for 29 days contained 127 mg of nitrogen and 41 mg of methionine as against 154 mg and 50 mg, respectively, when their ration contained methionine (table 5).

It may be recalled that the loss of large quantities of nitrogen through the urine during the period of protein deprivation in the metabolism test was accompanied by an equivalent loss in body weight, and that the addition of methionine to the diet although not preventing these losses did decrease their magnitude. In addition, the quantity of methionine present per gm of dry fat-free gastrocnemius muscle was constant in

TABLE 6

Methionine content of the gastrocnemius muscle of rats analyzed at different intervals of the balance test following the feeding of either a nitrogen-poor or a nitrogen-supplemented ration in period II.

INTERVAL OF BALANCE TEST	METHIONINE/GM DRY FAT-FREE MUSCLE
	mg
Beginning of experiment	38
End of period I; N-low diet, 18 days	36
End of period II	
N-low diet, 29 days	37
Egg supplement, 18 days	35
Me supplement, 18 days	
1 mg Me N/day	38
2 mg Me N/day	36
4 mg Me N/day	38
8 mg Me N/day	38
16 mg Me N/day	38

all experimental groups (table 6). These observations suggest that when methionine is not provided in the diet, the animal cannot selectively remove methionine from its tissues to meet its requirement for the amino acid. Instead it breaks down complete protein units to secure this vital substance, and throws away in the urine a large portion of the remaining molecule.

SUMMARY AND CONCLUSIONS

A marked reduction in the quantity of nitrogen excreted in the urine by standardized rats partially depleted of their bodily reserves of protein follows the introduction of whole

eggs into a protein-free ration. This effect may be ascribed to the amino acid content of the supplement. It has been shown that the influence of an equivalent quantity of nitrogen supplied in the form of the 10 essential amino acids is as marked as that of eggs providing 3.5% of dietary protein. Depressions in the excretion of urinary nitrogen lead to a marked sparing of body tissue. The validity of the concept, "body nitrogen spared," has been tested by relating changes in the nitrogen balance in the 2 metabolism periods to changes in body weight.

Cystine, choline, and all of the essential amino acids except phenylalanine, valine, and tryptophane exert some action in sparing body nitrogen. Of the group, methionine is the most powerful in this respect. No single amino acid, however, can be very important quantitatively in sparing body tissue in the partially depleted animal, in view of the complex demands of the body for the synthesis of its structural and functional components. Egg proteins and a mixture of the essential amino acids are much more effective in this respect than is any individual amino acid, as indicated by the quantities of body nitrogen spared and by restoration of weight loss when the nitrogen-poor ration was fed.

Analyses of whole carcasses, liver, and muscle of adult rats suggest that the effect of methionine in nitrogen metabolism is specific. Continued loss in body weight, the lack of change in the total nitrogen content of the carcass, the constancy of the ratio of methionine nitrogen to total nitrogen of the carcass, and the increment in hepatic tissue when methionine is fed to the depleted animal point to the possibility that this amino acid does not act in the general maintenance of body tissues but in the synthesis of functional proteins and important metabolites.

LITERATURE CITED

ALBANESE, A. A., J. FRANKSTON AND V. IRBY 1944 The estimation of methionine in protein hydrolysates and human urine. J. Biol. Chem., *156*: 293.

ALLISON, J. B., J. A. ANDERSON AND R. D. SEELEY 1945 The determination of the nitrogen balance index in normal and hypoproteinemic dogs. Preprinted manuscripts, Conference on proteins and protein hydrolysates in nutrition, The New York Acad. Sciences, Dec. 7 and 8.

BURROUGHS, E. W., H. S. BURROUGHS AND H. H. MITCHELL 1940 The independence of the endogenous and the exogenous metabolism of nitrogen. J. Nutrition, *19:* 271.

CROFT, P. B., AND R. A. PETERS 1945 Nitrogen loss after thermal burns, Lancet (London), *248:* 266.

FOLIN, O. 1905 A theory of protein metabolism. Am. J. Physiol., *13:* 117.

LAVINE, T. F. 1943 An iodometric determination of methionine. J. Biol. Chem., *151:* 281.

LEPKOVSKY, S., E. ROBOZ AND A. J. HAAGEN-SMIT 1943 Xanthurenic acid and its role in the tryptophane metabolism of pyridoxine-deficient rats. J. Biol. Chem., *149:* 195.

MADDEN, S. C., AND G. H. WHIPPLE 1940 Plasma proteins: their source, production and utilization. Physiol. Rev., *20:* 194.

MILLER, L. L. 1944 The metabolism of *dl*-methionine and *l*-cystine in dogs on a very low protein diet. J. Biol. Chem., *152:* 603.

MITCHELL, H. H. 1924 A method of determining the biological value of protein. J. Biol. Chem., *58:* 873.

MULFORD, D. J., AND W. H. GRIFFITH 1942 Choline metabolism. J. Nutrition, *23:* 91.

ROBSCHEIT-ROBBINS, F. S., AND L. L. MILLER 1946 Amino acid utilization in simultaneous hypoproteinemia and anemia. Elimination of 1 essential from growth mixture. Proc. Fed. Am. Soc. Exp. Biol., *5:* 226.

SAHYUN, M., EDITOR 1944 Outline of the Amino Acids and Proteins. Reinhold Publ. Corp., New York, N. Y., p. 225.

SCHOENHEIMER, R. 1942 The Dynamic State of Body Constituents. Harvard University Press, Cambridge, Mass.

SWANSON, P. P., W. WILLMAN, M. BRUSH, E. F. BROWN AND G. F. STEWART 1947 The "biological efficiency" of egg proteins. Submitted for publication as a Research Bulletin of the Iowa Agr. Exp. Station.

WILLMAN, W., P. P. SWANSON, G. F. STEWART, GLADYS T. STEVENSON AND M. BRUSH 1945 Biological efficiency of egg proteins. Proc. Fed. Am. Soc. Exp. Biol., *4:* 164.

WOLF, P. A., AND R. C. CORLEY 1939 Significance of amino acids for the maintenance of nitrogen balance in the adult white rat. Am. J. Physiol., *127:* 589.

COVITAMIN STUDIES

VI. EFFECT OF TOCOPHEROL SUPPLEMENTATION ON THE OUTPUT OF VITAMIN A, CAROTENE, AND FAT BY DAIRY COWS [1]

PHILIP L. HARRIS, WILLIAM J. SWANSON AND K. C. D. HICKMAN
Research Laboratories, Distillation Products, Inc., Rochester, New York

SIX FIGURES

(Received for publication October 18, 1946)

After it had been shown that vitamin E spares vitamin A in the economy of the rat (Harris et al., '44; Hickman et al., '44a, b), it seemed to us that the dairy cow would be the best animal for continuing the experiments. The cow derives vitamin A solely from the carotene of her feed, and the efficiency of utilization, judged by the final transfer to the milk (about 3%), is notoriously low. If the vitamin status or general health of the cow — particularly of barn-fed animals in wintertime — could be benefited by extra vitamin E, this simple procedure would ultimately have wide repercussions in human nutrition.

At the start of the project we believed that both carotene and vitamin A would be found in higher concentrations in the milk of animals given vitamin E. Vitamin A has been fed to cows by various experimenters in the hope of achieving the same end (Deuel et al., '42). Accordingly, we included vitamin A and mixed A and E supplementation in our study. Our preconceived objectives were not realized but we found such a significant increase in the production of butterfat and such an improvement in the health of the herds that we continued the experiment with open mind.

[1] Communication no. 102 from the laboratories of Distillation Products, Inc., Rochester, New York.

The technique of using cows on farms in our vicinity proved extraordinarily difficult and it was only after several failures that we began to get trustworthy results. For instance, in our earliest study the farm manager observed that the cows in one of the groups after about 6 weeks of vitamin E supplementation had much better appetites and healthier looking hair and hides. [illegible] ansferred all the groups to tocopherols without telling [illegible] At another farm the veterinarian gave our experimental animals intramuscular injections of vitamin A, without our knowing it.

After various experiences of this kind, we finally arranged for the use of a herd of 75 registered Brown Swiss cows.[2] We had complete control of the diet and did all supplement feeding, milk sampling and record keeping. The only restriction made by the owners was that we refrain from taking frequent blood samples, with which we had hoped to follow the vitamin concentration in the plasma parallel with that in the milk.

The form in which the vitamin supplements were fed proved to be of considerable importance. Merely placing measured volumes of oil concentrates of vitamin A or E on the feed in the manger was unsatisfactory. Some cows nosed the oily feed aside and refused it entirely while others ate only part of the supplement. Parenteral administration was not desirable because of the relatively poor utilization of fat-soluble vitamins when injected as compared with oral administration. Simple adsorption of the vitamin concentrates on flour or small portions of grain mixtures allowed for easy feeding but the vitamins proved to be unstable exposed in this way. The problem was finally solved by the use of specially manufactured powdered concentrates of vitamins A and E which had been stabilized against destructive oxidation.[3] Measured quantities of these dry vitamin concentrates were spread on

[2] Mr. E. Merrill and Mr. F. Roberts of Forest Farms, Webster, New York, were exceedingly cooperative and helpful in permitting the use of their dairy herd.

[3] "Myvadry," manufactured by Distillation Products, Inc.

the grain mixture once daily. In no instance did the animals refuse to consume the supplements completely.

Milk produced by each cow was carefully weighed at each milking on a Chattillon balance and the amount recorded. It was analyzed for butterfat twice each month, once by the licensed state tester and once in our own laboratory. The official Babcock test was used. Milk samples representative of the day's total output were analyzed every 2 weeks or oftener for vitamin A and carotene by the method of Koehn ('40). Calibration curves were constructed using a standard vitamin A ester concentrate and crystalline β-carotene. Consequently, analytical results were determined in terms of micrograms of vitamin A and of carotene. Although this is the preferable method of reporting results we have converted the values to "international units" to facilitate comparison with published figures. To convert micrograms of vitamin A to international units the conversion factor 3.5 was used. A factor of 1.67 was employed to change micrograms of carotene to international units. The calculation of "4% milk" was made using Gaines' formula ('28) namely "(pounds milk produced $\times$ 0.4) + (pounds fat produced $\times$ 15.0) = pounds 4% milk."

The cows were grouped insofar as possible according to age, stage of lactation, and relative amount of milk produced. They were fed grain throughout the year, even while on pasture from May through August. During the rest of the year they received corn silage and chopped, field-cured hay. Their feed was analyzed occasionally for carotene content and found to vary considerably. As a reasonable average value for vitamin A intake during stall feeding we used 550,000 units per day, of which approximately 40% was furnished by silage, 1% by the grain mixture, and 59% by hay.

Effect of vitamin E on fat concentration of milk and on milk output

It became evident in our preliminary experiments that the cows receiving extra amounts of vitamin E secreted milk with higher fat concentration. This increase was fairly large and

regular. Conversely, the volume of milk secreted by the vitamin E supplemented cows was usually slightly reduced. However, the overall change in production on the basis of "4% milk" was almost invariably an increase. An experiment was therefore set up to test the significance of the increase of

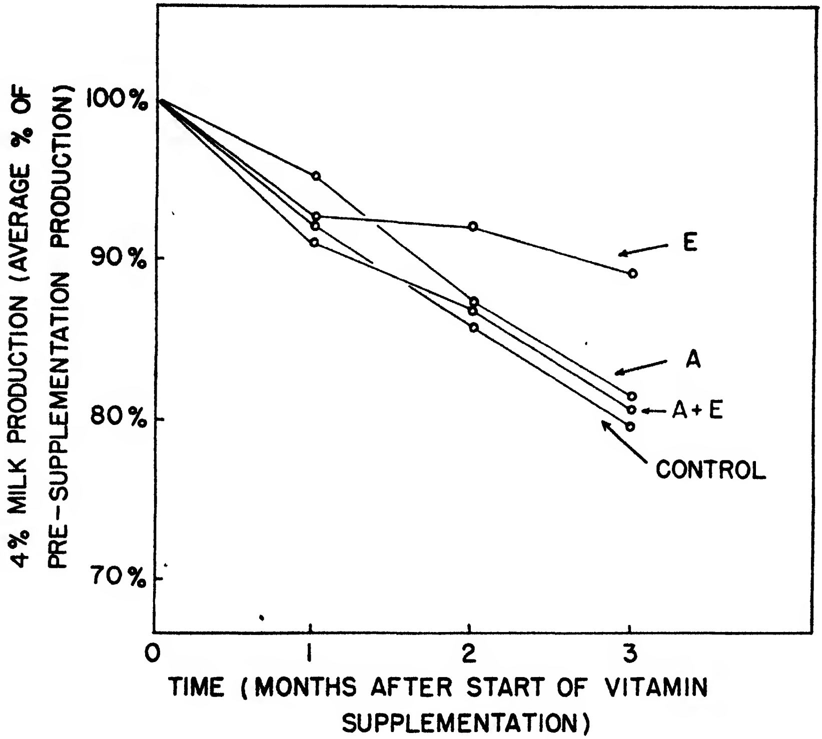


Fig. 1 Effect of vitamins A and E supplementation on the output of 4% milk by matched groups of Brown Swiss dairy cows.

"4% milk" production. Four groups were selected, each containing 4 carefully matched cows, and each group was supplemented in a different way with vitamins as shown in figure 1. The cows receiving vitamin E for more than 1 month of feeding maintained their milk production above the usual rate of decline. Cows receiving vitamin A and, surprisingly, vitamin A plus vitamin E did not show this effect. The cows

in this experiment were not used after 3 months of supplementation because several in each group had passed their ninth month of lactation.

Following this confirmation that vitamin E increased milk and fat output the experiment was repeated using a larger

TABLE 1

Effect of vitamin E supplementation on butterfat concentration.

NO. OF COW	AVERAGE BUTTERFAT % DURING PREVIOUS LACTATION (300 DAYS)	FAT CONCENTRATION OF MILK PRODUCED (%) 1[1]	2	3	4	5	6
		Group I control					
1A3	4.14	3.3	3.2	3.6	4.0	3.8	3.5
1B4	4.14	2.7	3.0	2.1	3.2	4.9	2.9
1C3	3.44	3.3	3.2	3.8	3.5	3.7	4.0
1D6	4.38	4.2	3.5	3.8	3.7	3.8	2.3
1E7	3.66	5.0	3.4	2.5	3.5	3.1	3.4
1F1	4.21	2.5	2.8	4.3	3.1	4.0	3.8
1G6	4.38	4.2	3.5	3.8	3.7	3.8	2.3
Monthly average	4.05	3.60	3.23	3.41	3.53	3.87	3.17
Total average				3.47			
		Group IV vitamin E supplement					
4A31	4.44	4.4	4.4	4.2	4.3	4.0	4.3
4B34	4.71	5.0	5.0	4.5	3.7	4.3	4.2
4C30	3.87	4.6	4.7	5.1	4.6	4.3	4.5
4D35	4.35	5.5	4.7	4.7	4.2	4.0	4.0
4E36	3.97	3.6	3.3	3.5	3.6	3.5	3.7
4F34	3.87	5.0	4.5	3.7	4.3	4.2	3.7
4G33	3.87	5.3	5.3	5.2	5.2	5.6	5.4
Monthly average	4.15	4.77	4.56	4.41	4.27	4.27	4.26
Total average				4.42			

[1] Figures refer to calendar months during experiment.

number of matched pairs of cows which were not so far advanced in lactation. One group was used as controls while the animals of the other group were fed 1.0 gm extra of natural mixed tocopherols daily. The findings are shown in tables 1 and 2. Analyses of variance (Bliss and Marks, '39) of these

data confirm the fact that both butterfat concentration and "4% milk" production are significantly ($P = < 0.05$) increased by tocopherol supplementation.

TABLE 2

Effect of vitamin E supplementation on output of "4% milk."

NO. OF COW	AVERAGE PRODUCTION DURING PREVIOUS LACTATION (300 DAYS)	PRODUCTION OF "4% MILK" (LBS./DAY)					
		1[1]	2	3	4	5	6
		Group I control					
	(lbs./day)						
1A3	37.62	37.03	36.64	32.19	34.96	30.51	27.48
1B4	30.51	29.57	33.09	24.98	29.90	25.65	20.60
1C8	37.07	37.00	36.60	30.51	27.50	26.47	27.13
1D9	25.61	46.18	42.91	34.40	34.56	30.42	29.85
1E7	36.71	57.73	50.74	38.41	39.90	34.01	31.28
1F1	37.66	36.64	35.47	45.11	31.07	30.84	28.29
1G6	25.61	52.20	44.40	43.70	40.24	34.60	29.50
Monthly average	32.97	42.34	39.98	35.61	34.02	30.36	27.73
Total average	32.97	35.01					
		Group IV vitamin E supplement					
4A31	27.13	42.63	47.93	45.08	42.80	36.52	36.46
4B34	34.04	62.10	59.80	53.79	51.06	44.36	40.59
4C30	21.86	34.34	28.31	31.07	32.34	30.45	30.96
4D35	33.90	61.90	59.51	57.86	54.58	51.38	54.81
4E36	30.93	32.59	34.25	33.91	31.91	31.82	28.83
4F38	34.04	59.80	53.79	50.06	48.36	40.59	36.12
4G39	30.30	41.14	38.40	36.78	34.06	32.78	33.23
Monthly average	30.31	47.79	46.00	44.08	42.16	38.23	37.29
Total average	30.31	42.59					

[1] Figures refer to calendar months during experiment.

It should be pointed out that the experiment from which the data in tables 1 and 2 were obtained was conducted throughout the 1-year period from February, 1945, to February, 1946. The supplements were given during the summer months of pasture feeding as well as during the winter months of stall feeding.

Unexpectedly, the effects of additional tocopherol persisted even through the period of pasture feeding.

In general, the increase in "4% milk" output due to tocopherol supplementation varies from 5 to 15%. However, the increase in fat concentration induced by tocopherol ranges between 10% and 40%. Figure 2 shows the butterfat levels in 3 matched groups of 6 cows each, studied for 21 months. It is evident that the additional tocopherol has induced a metabolic change which is reflected in an increase in fat concentration in the milk. That this effect is rather rapid was

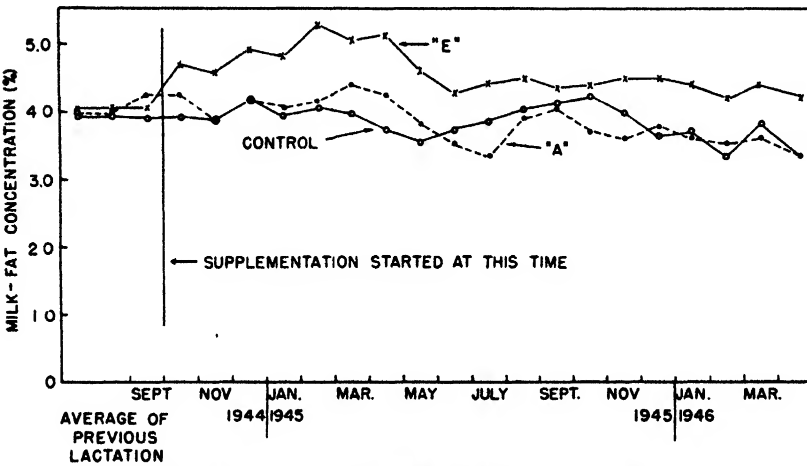


Fig. 2 Changes in fat test of dairy cows induced by vitamin supplementation.

demonstrated by several tests of the cross-over type. Individual cows in the vitamin E supplemented group ceased receiving a supplement, and cows in the control group were given vitamin E. Ten days after the changes were made there were noticeable changes in fat concentration in the milk. Upon returning to the original feeding program the fat concentrations were reversed, again within 10 days.

Further confirmation of this effect of vitamin E on milk-fat production was obtained in a short experiment at the Silver Forest Farm in Forestville, New York.[4] Four closely

[4] We appreciate the kindness of Mr. L. Hawkins and Mr. I. Dodge in allowing us to report these data.

matched trios of registered Guernsey cows were used. The groupings and production data are shown in table 3. Cow 1 in each trio was used as the control and received no supplement. Cow 2 received 1.0 gm of vitamin E daily in the form

TABLE 3

Output of "4% milk" by Guernsey cows as affected by vitamin E supplementation.

	COWS [1]	GROUP OR TRIO NUMBER 1	2	3	4	AVERAGE
	1	12-39	1-39	3-43	6-43	
Date of birth	2	8-41	1-40	7-42	1-43	
(month and year)	3	10-42	12-37	6-43	8-42	
	1	4	4	1	1	
Lactation	2	3	4	2	1	
	3	2	6	1	2	
	1	11-45	11-45	11-45	11-45	
Date of freshening	2	12-45	11-45	11-45	10-45	
	3	12-45	11-45	12-45	10-45	
Production during	1	47.2	46.2	30.3	40.0	
January, 1946	2	54.5	57.5	33.2	22.6	
(average lbs. milk/day)	3	50.8	51.3	32.1	24.8	
Milk-fat concentration	1	4.30	5.58	5.70	4.97	
during January, 1946	2	5.52	4.68	4.00	4.90	
(average % fat in milk)	3	4.59	4.65	5.26	4.90	
4% milk produced during	1	49.33	57.17	38.09	45.85	
January, 1946	2	60.95	63.35	33.20	25.70	
(lbs.)	3	55.25	56.37	38.18	28.06	
Change in production of	1	—18.0	—26.6	—26.0	—14.1	—21.2
4% milk, compared with	2	—10.9	—14.3	—14.3	—5.8	—11.3
that of Jan., in Feb. (%)	3	—9.4	—1.8	—14.2	—9.1	—8.6
	1	—10.9	—24.8	—26.1	—16.0	—19.5
Change in March (%)	2	—7.7	—16.7	—19.2	—4.4	—12.0
	3	—1.7	—12.8	—22.7	—8.5	—11.4
	1	—23.9	—32.8	—36.8	—20.9	—28.6
Change in April (%)	2	—9.6	—20.0	—24.8	—10.0	—16.1
	3	—12.2	—25.1	—23.6	—11.6	—18.1

[1] Cow 1 in each trio received no supplement and was the control.

Cow 2 in each trio received 1.0 gm of mixed tocopherols, of which 10% was in the α- form.

Cow 3 in each trio received 1.0 gm of tocopherols, of which 60% was in the α- form.

of a concentrate of mixed natural tocopherols, chiefly γ- and δ-tocopherol with 10% α-tocopherol. Cow 3 received the same amount of vitamin E in the form of a concentrate of mixed natural tocopherols (60% α-tocopherol) similar to that used at Forest Farms. The supplements were begun February 1, 1946. The production level for January, 1946, for each cow was used as the base value and changes from this constituted the criterion of response due to supplementation. Figure 3

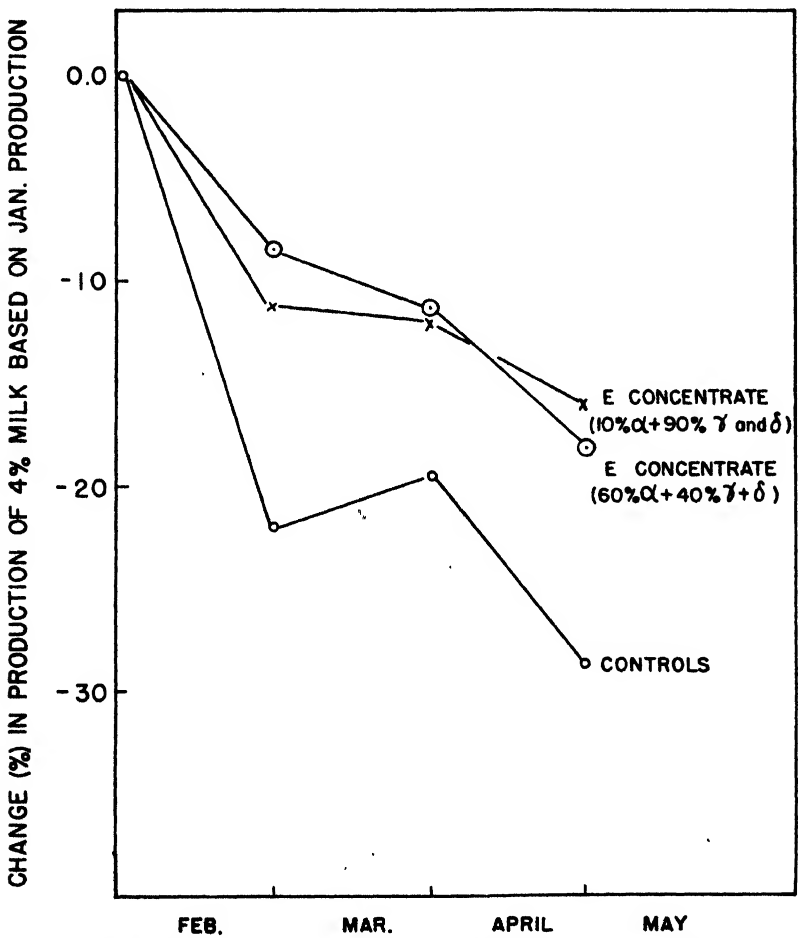


Fig. 3 Effect of vitamin E on the output of 4% milk by matched groups of Guernsey cows.

shows these changes and indicates that: (1) vitamin E beneficially influenced "4% milk" production in Guernsey cows, and (2) the γ- and δ-tocopherol concentrate low in α-tocopherol seems equally effective for this purpose. The differences in "4% milk" production recorded in figure 3 between the control group and the 2 vitamin E supplemented groups were statistically significant ($P = < 0.05$).

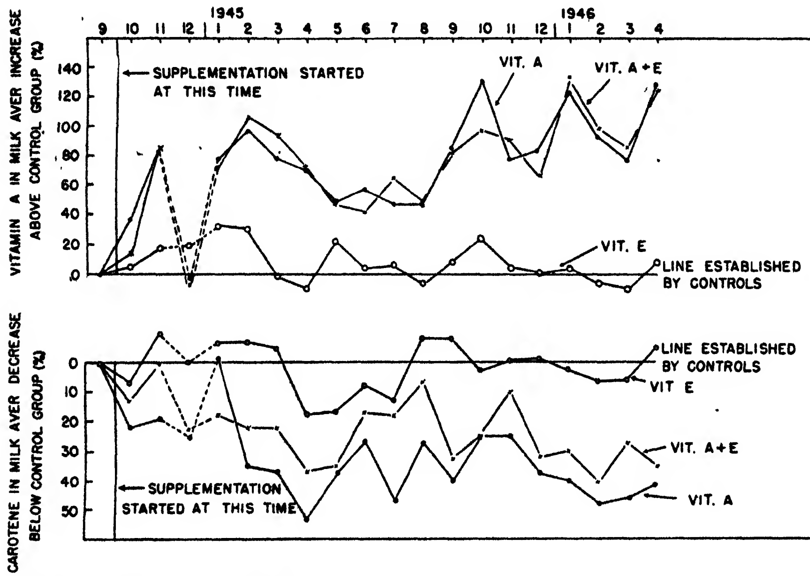


Fig. 4 Changes in concentration of vitamin A and carotene in milk due to vitamin supplementation. The dashed portions of the curves indicate a period of 2 weeks in December, 1944, during which the cows were not supplemented.

Effect of vitamins A and E on concentration of vitamin A and carotene in milk

Data for this are shown in figure 4. It seems obvious that insofar as the concentration or the total output of vtamin A *per se* is concerned, vitamin E caused no increase. The average increase in vitamin A above the controls for both the A and the A + E groups is about 75%. The vitamin E group averaged about 10% higher in vitamin A concentration of milk than the control group, but since the increase occurred all

at the beginning of the period as shown in the curves in figure 4 it is not considered significant.

The effect of vitamin E on carotene concentration in the milk was somewhat different. It is now well established that large doses of vitamin A depress the level of carotene in the blood and milk of dairy cows (Deuel et al., '42). This is confirmed in figure 4 where the carotene level of the vitamin A group is 33% below that of the control group. However, when vitamin E was fed simultaneously with the vitamin A the depression of carotene was not so pronounced; it was about 23% lower on the average than that of the control group. Vitamin E alone did not affect the carotene of the milk. There is rather great variability of response in these experiments, but since the trend is constant and has been confirmed in shorter tests it seems reasonable to conclude that when vitamin E is administered with vitamin A the carotene concentration in the milk is lowered only two-thirds as much as when vitamin A is fed alone.

The overall efficiency of transfer [5] of carotene from feed to vitamin A potency in the milk is shown in figure 5. The formula used is as follows:

$$\text{``Efficiency of transfer''} = \frac{\text{I.U. secreted by supplemented cows} - \text{I.U. secreted by control cows}}{\text{I.U. of A in daily supplement}}$$

The bar graph at the top of figure 5 shows the apparent variation in efficiency of transfer over a 15-month period. First, however, it should be noted that the overall average efficiencies for the A-fed group and the group fed A + E are essentially the same, about 2.4%. The efficiency percentages vary during the year from as much as 6% in the winter (January and February) during stall feeding to less than 1% in the summer (June and July) when fresh pasturage is available.

The absolute values and seasonal variations for the average concentration of vitamin A potency (vitamin A *per se* plus

[5] I.U. secreted equals the sum of the carotene and the vitamin A in international units contained in the milk secreted daily.

carotene) in the milk of the control group are also of interest (fig. 6). For example, the maximum average concentration (approximately 1,800 units per quart) occurs in the period from June through October and rapidly drops to a minimum (800 to 900 units per quart) in the period from January through April. This means, of course, that the milk consumer may obtain milk in the winter which furnishes him with

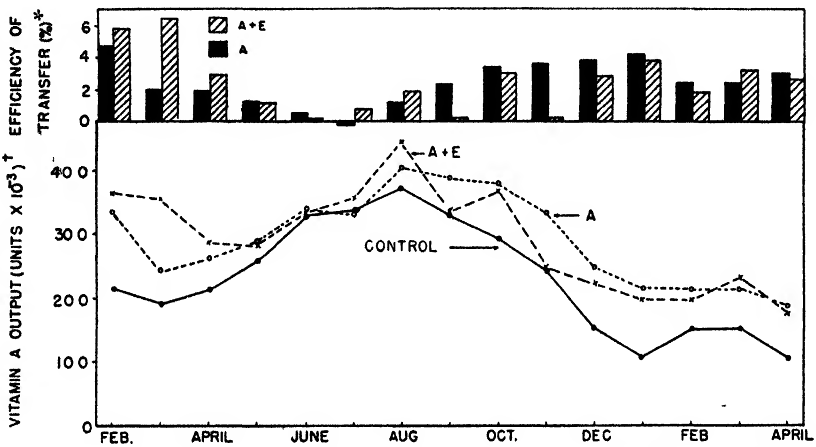


Fig. 5 Influence of vitamin A vs. vitamin A and E in effecting transfer of vitamin A potency into the milk.

† Average number of I.U. of vitamin A potency secreted per day per cow (vitamin A + carotene). Increase in vitamin A potency (vitamin A + carotene) of milk related to vitamin A in daily supplement.

* Increase in vitamin A potency (vitamin A plus carotene) of milk related to vitamin A in daily supplement.

only 50% of the vitamin A potency of summer milk. These variations are also shown in figure 6, expressed on the basis of butterfat. Carotene contributed about 34% of the total vitamin A activity of the milk (Lord, '45). This varied from 25% in the winter to 40% in the summer.

Referring again to figure 5, it is evident that supplementation of the dairy cow with 250,000 units of vitamin A per day during the winter is not enough to maintain the vitamin A potency of the milk at the summer level. Deuel and co-workers ('41) have previously noted this and state that the diet of the

cow must be supplemented with at least 700,000 units of vitamin A to achieve a significant increase in vitamin A concentration in the milk. In experiments not reported in detail in this communication, we have fed vitamin A supplements of 500,000, 750,000, and 1,000,000 units per day. The 2 higher levels were necessary to maintain the vitamin A potency at summer values. This, of course, would not be economical as a means of supplying vitamin A to the consumer. It should be done only if there is evidence that it improves the general health of the cow.

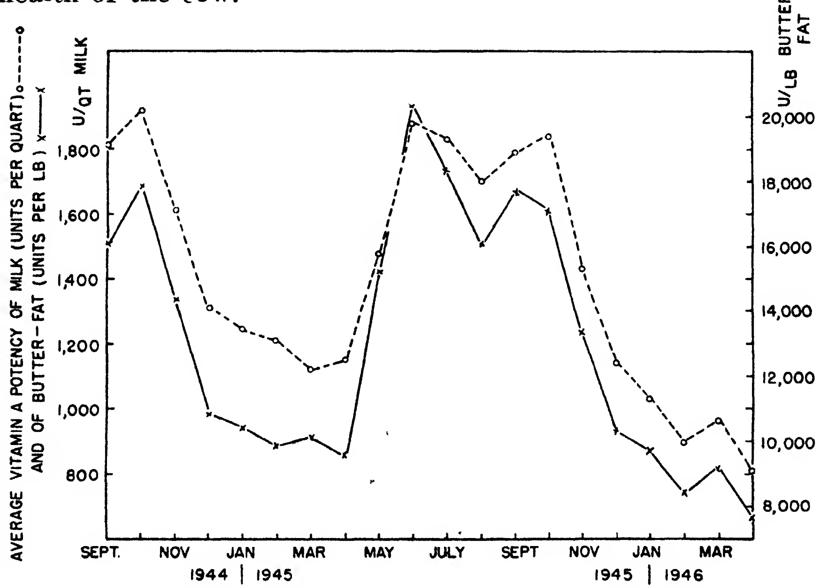


Fig. 6 Changes in total vitamin A potency of milk and of butter fat of Brown Swiss control cows.

General effects of vitamin E supplementation

Impressions and opinions of the herdsman and farm manager, although subjective in nature, are also of considerable value in evaluating the responses to vitamin E supplementation. These men reported that the supplemented cows had increased appetites. They also noted that the cows looked healthier and more vigorous. Generalized infections were

completely absent. There was less mastitis (strip-cup test) than in the controls or in the herds of previous years according to the records. The ratio of services to conceptions decreased from 5 to 1 in previous years to less than 3 to 1 among the supplemented cows. There were no stillbirths and only 1 abortion. On only 1 occasion was it necessary to remove the fetal membrane following parturition. The record for the previous year showed 3 stillbirths, 4 abortions, and retained placentas requiring the services of a veterinarian after the birth of almost every calf. In general, our previous experience with other herds, which indicated that supplementation with vitamin E or with both vitamins A and E is highly desirable, has been confirmed in this investigation.

DISCUSSION

The benefits to dairy cows of supplementation with vitamin E (and A) may be due in part to the effect of these vitamins on the microorganisms of the rumen. Vitamin E in particular may affect the oxidation-reduction potential of rumen contents enough to change the proportions of the various organisms. We considered the possibility of this mode of action of vitamin E upon finding that very little of the administered tocopherol was secreted in the milk. For example, the tocopherol content of summer milk-fat was 42 μg per gm for control cows and between 49 and 53 μg per gm for E-fed animals. Winter milk-fat contained about 23 μg per gm and 47 μg per gm for control and E-fed cows, respectively. Some of the tocopherol apparently found its way into the body of the cow and was secreted into the milk. The efficiency of this transfer is very low; 2% would seem to be a maximum value. However, it should be kept in mind that we do not know the optimum dose of tocopherol for cows. All of these experiments were carried out using 1.0 gm of tocopherol. We have yet to investigate the responses at different dosage levels.

The vitamin E concentration in the blood of the cows was determined only at 1 time of the year, during February. The

average for the control group was 0.45 mg % and for the E-fed group 1.06 mg %. In previous studies on Holstein cows the tocopherol levels in the blood of control and E-fed cows showed the same difference. The tocopherol level considered normal for humans is 1.0 mg or more per 100 ml of plasma (Quaife and Harris, '44), and it is interesting to speculate that perhaps the tocopherol supplement was necessary to raise the vitamin E nutriture of the cow to normal.

An interesting sidelight of this study concerns the difference in curd formation in the milk from the various groups of cows. This will be investigated in detail as soon as possible. In brief, the milk from E-fed cows coagulates less quickly than milk of control cows, while the milk of A-fed cows coagulates more quickly. Coagulation is considered as visible clotting of the protein and separation of the milk into whey and clot. Surprisingly enough, the increase in titratable acidity is the same in all of the milks, but invariably the milk from the A-fed cows separates more quickly and that from the E-fed cows less quickly than that from the control cows. It is not impossible that some fundamental change in the proteins of the milk has taken place under the influence of either vitamin A or vitamin E which would account for the difference in clotting.

SUMMARY AND CONCLUSIONS

A herd of purebred Brown Swiss dairy cows divided into 4 comparable groups has been studied for a period of 20 months. Three groups received daily supplements of: (1) 250,000 units of vitamin A, (2) 1.0 gm of mixed natural tocopherols, and (3) both vitamin A and tocopherols, and a fourth group received no supplement. Milk production, fat concentration in the milk, total fat production, and output of vitamin A and carotene in the milk were studied extensively. Shorter experiments were conducted on the tocopherol content of the blood and milk of the cows.

Tocopherol supplementation increased milk-fat concentration about 27% and total milk production ("4% milk") about

21%. The same response was induced in Guernsey cows fed vitamin E. Only cows in the same stage of lactation and as nearly matched as possible in other respects, such as age and number of lactations, were compared.

Supplementation with vitamins A and E was no better than with vitamin A alone in increasing the vitamin A *per se* in the milk. However, the decrease in carotene concentration in the milk due to the feeding of vitamin A was less severe (approximately 23% below the control level rather than 33%) with the simultaneous administration of vitamin E.

The efficiency of transfer of ingested vitamin A and carotene into vitamin A and carotene in the milk was not increased by supplementation with vitamin E alone. The average value for a 1-year period was 2.4%. This varied from about 6% in the winter under barn-feeding conditions to less than 1% in the summer when fresh pasture was available. The total output of vitamin A potency (vitamin A plus carotene) of the control cows varied from a maximum value in August to 30% of this in January. Carotene contributed about 34% of the total vitamin A activity of the milk, and this varied from 25% in winter to about 40% in summer. General improvement in appearance, reproduction, and health was noticed in the parts of the herd supplemented with vitamin E and vitamins A and E.

Finally, in comparing the benefits to be derived from the 3 types of supplementation, E, A, and E + A, it must be concluded that the major benefit in health and milk output was derived from vitamin E alone. The only obvious result of vitamin A supplementation was to increase the vitamin A content of the milk, this at the partial expense of carotene content. The combined supplementation of E and A allowed the A content of the milk to rise without a pronounced fall of carotene, but the increase in butterfat which we have come to expect from vitamin E supplementation was entirely repressed by the vitamin A. Vitamin E therefore emerges as the preferred supplement to the ordinary diet of dairy cows.

LITERATURE CITED

BLISS, C. I., AND H. P. MARKS 1939 The biological assay of insulin II. The estimation of drug potency from a graded response. Quart. J. Pharm. Pharmacol., *12:* 182.

DEUEL, H. J., JR., N. HALLIDAY, L. F. HALLMAN, CORNELIA JOHNSTON AND A. J. MILLER 1941 Production of high vitamin A milk by diet. J. Nutrition, *22:* 303.

DEUEL, H. J., JR., L. F. HALLMAN, C. JOHNSTON AND F. MATTSON 1942 Effect of high vitamin A intake on blood and milk carotene of Holstein and Guernsey cows. J. Nutrition, *23:* 567.

GAINES, W. L. 1928 The energy basis of measuring milk yield in dairy cows. Univ. of Ill., Agr. Expt. Sta. Bull. 308.

HARRIS, P. L., M. W. KALEY AND K. C. D. HICKMAN 1944 Covitamin studies II. The sparing action of natural tocopherol concentrates on carotene. J. Biol. Chem., *152:* 313.

HICKMAN, K. C. D., M. W. KALEY AND P. L. HARRIS 1944a Covitamin studies I. The sparing action of natural tocopherol concentrates on vitamin A. J. Biol. Chem., *152:* 303.

——— 1944b Covitamin studies III. The sparing equivalence of the tocopherols and mode of action. J. Biol. Chem., *152:* 321.

KOEHN, C. J. 1940 A rapid photoelectric method for the determination of vitamin A and carotene in milk. J. Biol. Chem., *133:* lvi.

LORD, J. W. 1945 Seasonal variation of carotene and vitamin A in butterfat and in serum. Biochem. J., *39:* 372.

QUAIFE, M. L., AND P. L. HARRIS 1944 The chemical estimation of tocopherols in blood plasma. J. Biol. Chem., *156:* 499.

SOME OBSERVATIONS ON THE NUTRITIONAL VALUE OF DIALYZED WHEY SOLIDS

F. K. DANIEL AND E. H. HARVEY

Food Division, Sun Chemical Corporation, Long Island City, New York

(Received for publication November 1, 1946)

Lactalbumin is known to be a more nearly complete protein and in biological studies has proved to be a far more efficient source of dietary nitrogen than casein (Weech, '42; Melnick, Cowgill and Burack, '36; Melnick and Cowgill, '37). It is also known that human milk is much higher in lactalbumin than cow's milk. The reported composition of average human milk varies from about 1.0% lactalbumin and 0.5% casein (ratio 2:1) (Williamson, '44) to about 0.75% lactalbumin and 0.5% casein (ratio 3:2), the latter being the most widely accepted figure (McLester, '39; Jeans, '43). Cows' milk runs fairly constant at about 0.5% lactalbumin and 3.0% casein (ratio 1:6). It would seem logical that an infant food of a similarly high lactalbumin/casein ratio as that of human milk should have a higher nutritional value than one based on cows' milk alone.

A formula of this kind, high in lactalbumin, was devised by the partial substitution of whey for milk proteins. Comparative feeding tests showed unexpectedly, however, that the rats on the normal milk (low lactalbumin) diet gained more on slightly less food intake than the rats on the high lactalbumin diet. This is the reverse of what had been expected. It was the object of this investigation to clear up the apparent discrepancy between the higher biological value of lactalbumin and the poorer results in actual feeding when the lactalbumin is incorporated into the formula by the addition of whey solids.

EXPERIMENTAL

Feeding tests were carried out with male albino rats of the Sprague-Dawley strain, 21 days of age and with a 5-gm weight range. They were placed on individual raised screen cages and fed the experimental diets for 6 weeks. Food and water were given ad libitum and food consumption records were kept for the group as a whole. Six animals were fed each ration. Weekly weight gains were recorded for each rat and the mean as well as standard deviation for the 6-week gains reported in the results. This same procedure was followed throughout all the experiments. The vitamin and mineral supplement was identical in all diets. The protein level in all experiments was held below that for optimal rat growth, viz., at 12% and in this as in all other respects the diets closely approximate the composition of human milk. The data are summarized in tables 1 and 2.

Test no. 1

This was a comparison of a diet based on milk proteins only (Control) with a high lactalbumin diet containing milk plus whey proteins (Test). (Columns 1 and 2 in table 1.)

The increase in rate of growth and the greater efficiency in converting the food into body tissue, both indicate a superior nutritional value of the control formula.

In the control diet the lactalbumin/casein ratio was about 1 : 6 and in the test diet it was 3 : 2 as in human milk. The fat content was identical in both formulae but the carbohydrate was slightly different. Four per cent carbohydrates were withheld from the test diet, to compensate for the high mineral content of the whey (table 2). This slight difference in carbohydrates was not deemed important.

During the experimental period, animals of both groups were normal and healthy in appearance, although, the animals receiving the test ration containing whey had diarrhea throughout the 6-week period.

TABLE 1

Comparative rat feeding tests with milk and dialyzed or undialyzed whey additive.

	TEST I		TEST II			TEST III	
	Milk proteins only	Milk and whey proteins	Undialyzed whey proteins	Dialyzed whey proteins	Dialyzed whey proteins plus Minerals	Milk proteins only	Milk proteins plus dialyzed whey proteins
	Control	Test	Control	Test	Check	Control	Test
	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7
Ration:							
Dry whole cows' milk (gm)	45.30	21.00	21.20	21.20	21.20	45.30	21.00
Undialyzed whey powder (gm)		57.70	57.70				
Dialyzed whey powder (gm)				44.00	44.00		44.50
Lactose (gm)	24.32			9.20	9.20	25.32	
Sucrose (gm)	13.24	0.20				13.24	13.24
Butterfat (gm)	15.10	21.20	21.10	21.27	21.27	15.10	21.26
Minerals (gm)	0.78 [1]				3.00 [2]	0.78 [1]	
Lactic acid (gm)	0.59			.40	.40	0.59	
Water (gm)	0.67			3.91	.91	0.67	
Composition:							
Protein (%)	12.00	12.00	12.05	12.03	12.03	12.00	12.00
Lactalbumin (%)	1.72	7.20	7.20	7.20	7.20	1.72	7.20
Casein (%)	10.28	4.80	4.85	4.83	4.83	10.28	4.80
Carbohydrate (%)	54.51	50.37	50.90	50.90	50.90	54.51	54.51
Lactose (%)	41.27	50.17	50.90	50.90	50.90	41.27	41.27
Sucrose (%)	13.24	0.20				13.24	13.24
Fat (%)	27.50	27.50	27.30	27.30	27.30	27.50	27.50
Minerals (%)	3.44	6.39	6.37	3.37	6.37	3.44	3.44
Lactic acid (%)	0.67	0.67	.99	.99	.99	0.59	0.59
Moisture (%)	0.70	2.70	2.60	5.60	2.60	0.67	1.80
Results:							
Average weight gained (gm)	99.00	91.00	102.00	122.00	102.00	99.00	125.00
Standard deviation (gm)	12.86	12.90	8.66	10.72	6.42	12.86	14.15
Standard deviation (%)	13.00	14.20	8.48	8.78	6.28	13.00	11.32
Daily food consumption (gm)	8.36	9.80	9.43	10.00	10.20	8.36	9.31
Weight of ration necessary for 1 gm gain in body weight (gm)	3.55	4.53	3.88	3.44	4.20	3.55	3.10
Galactose excretion (%)		.	12.38	6.83	14.00	18.89	8.48

[1] Osborn-Mendel mixture.

[2] Whey diffusate ash.

To determine the possible influence of the higher mineral content on the above results, it seemed desirable to introduce the lactalbumin into the diet free of minerals or at least with a greatly reduced mineral content. In the customary processes for separating lactalbumin from the other whey constituents, denaturization occurs and the resulting insolubility is undesirable in infant feeding formulae. To remove the minerals without denaturing the protein, a special commercial dialysis technique was worked out which reduced the soluble ash fraction from 5.46% to 1.30%.

Diets in which this dialyzed whey replaced undialyzed whey showed greatly improved growth response. In order to confirm this result, the fraction dialyzed out of the whey (diffusate) was collected, dried, ashed and was then reintroduced to the dialyzed whey to restore the original mineral concentration. On the assumption that the diffusate minerals are the factors responsible for the adverse effect on growth, the result of this test may be expected to be similar to that obtained with undialyzed whey, and this is confirmed by the test.

The ashing eliminates the possibility of an organic growth inhibiting factor being responsible, but without a qualitative and quantitative determination of the various mineral constituents removed by dialysis, the question remains unanswered which of the minerals is responsible for the adverse effect on growth.

Test no. 2

This was a comparison of diets containing undialyzed whey (Control), dialyzed whey (Test) and dialyzed whey with restored minerals (Check) columns 3, 4 and 5 of table 1.

The composition of the diets in all 3 groups is identical with the exception of the minerals.

The beneficial results of dialysis are evident from an inspection of the data. The rats on the dialyzed whey diet ate 6% in excess, and gained 19.6% more than those on undialyzed whey; in all they showed a 12.8% greater gain in body weight

per gm food intake than the rats on the undialyzed whey diet. The slight variation in the results of the control group and the check group may be due to some changes in chemical composition of the minerals brought about by the ashing at high temperature (700°F).

The galactose excretion was determined after 5 weeks on the experimental diet. During the experimental period, animals receiving all 3 diets were normal and healthy in appearance. Diarrhea was prevalent among the rats receiving the test ration only during the first week of experiment. Animals receiving the undialyzed whey ration and those receiving the dialyzed whey plus the ashed whey diffusate had diarrhea throughout the 6-week period.

TABLE 2

Proximate analysis of undialyzed and dialyzed whey.

	VALUES FOUND	
	Spray dried whey powder	Spray dried dialyzed whey powder
Total solids (%)	96.22	97.12
Moisture (%)	3.78	2.88
Ash (%)	8.93	4.93
Protein (N × 6.38) (%)	11.08	14.47
Fat (ether extract) (%)	1.09	1.05
Lactic acid (%)	1.71	1.33
Carbohydrate (by difference) (%)	73.41	75.34
Partition of the ash		
Total ash (%)	8.93	4.93
Insoluble ash (%)	3.47	3.63
Soluble ash (%)	5.46	1.30
Chloride (as Cl) (%)	1.75	0.24
Calcium (as Ca) (%)		0.81
Phosphorus (as P) (%)		0.67
Sodium (as Na) (%)		0.38
Tests on protein component		
Casein (%)		Less than 0.1
Lactalbumin + lactoglobulin (%)		14.4
pH	5.57	5.63
Cal./100 gm	355	374

Whey used in these experiments, both the dialyzed and the undialyzed, was from the same batch of cheddar cheese whey which was processed very carefully to avoid denaturization. The whey remained completely water soluble even after drying. The dialysis treatment was relatively brief, by no means exhaustive. Hence, the results reported here for the dialyzed whey may not necessarily be the most favorable ones.

The changes brought about by dialysis may be seen by the comparative analysis given in table 2.

The most significant difference between the 2 analyses is in the amount of soluble ash. The soluble ash is reduced out of proportion to the total ash because a certain fraction of the calcium and phosphorus is an integral part of the protein complex and cannot be removed by dialysis.

In the final test, a diet containing dialyzed whey was compared with an identical diet without whey, the only variation in composition being in the ratio of lactalbumin and casein. In order to have the same mineral content of 3.44% in each case, 0.78% minerals (Osborne-Mendel mixture) were added to the control diet.

Test no. 3

This was a comparison of a diet based on milk proteins only (Control) with a high lactalbumin diet containing milk plus dialyzed whey proteins (Test) (columns 6 and 7 in table 1).

The animals fed on the test diet ate 11.4% in excess, and gained 26.3% more than those on milk proteins; in all they showed a 14.5% greater gain in body weight per gm food intake than the rats on the control diet.

During the experimental period, animals in both groups were normal and healthy in appearance. Diarrhea was prevalent among the rats receiving the test diet during the first week and again during the sixth week.

CONCLUSIONS

In normal (undialyzed) whey the biological value of the lactalbumin is masked so that it appears to be less than that of casein. This may explain why whey is not now being used

in infant feeding formulae in spite of the recognized food value and desirability of its 2 chief constituents: lactose and lactalbumin, and the availability of whey at low cost. In dialyzed whey the true biological value of the lactalbumin becomes evident, demonstrating its superiority over casein by a considerable margin.

Mineral constituents of the whey are responsible for its low nutritional value, which is associated with lessened appetite, a tendency for diarrhea and an impaired ability to digest carbohydrates, as shown by the higher galactose excretions. The reason for, and the mechanism by which these changes are brought about, are not apparent from these tests.

Rats fed on the dialyzed whey formula showed greater appetite than the rats fed on a milk formula only.

Dialysis is capable of converting whey, a dairy by-product, into a highly valuable ingredient for infant formulae. Whether the degree of dialysis arbitrarily chosen here results in the best possible product for this or other purposes, remains to be determined by future experiments.

SUMMARY

1. Partial substitution of whey proteins for casein, in a formula resembling human milk, detrimentally affects the growth of rats.

2. Removal of a considerable portion of minerals from whey by means of dialysis results in a more favorable growth response. On a diet containing dialyzed whey, the rats grow 12.8% better per gm of food intake than on the same diet containing undialyzed whey.

3. If the minerals removed from the whey by dialysis are reintroduced to the dialyzed whey after ashing, the rats' growth again is impaired. This proves that 1 or more of the inorganic constituents, rather than an organic compound, affects detrimentally the nutritional value of whey.

4. On a diet in which 53% of casein is replaced by dialyzed whey lactalbumin, rats show 14.5% greater efficiency in conversion of food to body tissue than rats fed on a corresponding

formula containing milk proteins only. This experimental result is in agreement with previous work whereas the results with undialyzed whey seemed to dispute the well established biological superiority of lactalbumin over casein.

ACKNOWLEDGMENT

We wish to express sincere appreciation to Dr. C. A. Elvehjem and Mr. P. H. Derse, Department of Biochemistry, University of Wisconsin, for conducting the animal feeding tests reported in this paper.

To Messrs. A. C. Horn and A. E. Horn we are greatly indebted for financial support and encouragement, and to Mr. N. L. Simmons we are obliged for the use of his plant facilities.

LITERATURE CITED

JEANS, P. C. 1943 Feeding of Healthy Infants and Children. Handbook of Nutrition, American Medical Association, p. 340.

MCLESTER, J. S. 1939 Nutrition and Diet in Health and Disease. W. B. Saunders, Philadelphia, 3rd ed., p. 183.

MELNICK, D., AND G. R. COWGILL 1937 The protein minima for nitrogen equilibrium with different proteins. J. Nutrition, *13:* 401.

MELNICK, D., G. R. COWGILL AND E. BURACK 1936 The influence of diet upon the regeneration of serum protein. II. The potency ratios of serum protein, lactalbumin and casein, and the effect of tissue protein catabolism upon the formation of serum protein. J. Exp. Med., *64:* 897.

WEECH, A. A. 1942 Dietary protein and the regeneration of serum albumin. Bull. Johns Hopkins Hosp., *70:* 157.

WILLIAMSON, M. B. 1944 The amino acid composition of human milk proteins. J. Biol. Chem., *156:* 47.

NITROGEN RETENTION STUDIES ON RATS, DOGS AND MAN; THE EFFECT OF ADDING METHIONINE TO AN ENZYMIC CASEIN HYDROLYSATE

WARREN M. COX, JR., ARTHUR J. MUELLER, ROBERT ELMAN, ANTHONY A. ALBANESE, K. S. KEMMERER, R. W. BARTON, AND L. EMMETT HOLT, JR.

WITH THE TECHNICAL ASSISTANCE OF KATHRYN R. DRONE, DOROTHY SLOAT, MARILYN LEIN AND HARRIET W. DAVEY

Department of Nutritional Research, Mead Johnson and Company: Department of Surgery, Washington University School of Medicine and Barnes Hospital, St. Louis; and Department of Pediatrics, New York University and Bellevue Hospital, New York[1]

TWO FIGURES

(Received for publication November 22, 1946)

The literature is replete with evidence that casein contains but little cystine and that its nutritive value can be improved by the addition of small quantities of this amino acid. The early findings by Osborne and Mendel ('15) that casein would not support as rapid growth in rats at a 9% level as lactalbumin is the basis of the oft-cited nutritional superiority of mother's milk to cow's milk. Marriott and Jeans ('41) state: "Human milk contains less protein than cow's milk, but of the total protein present, almost 60% is lactalbumin, as compared with about 15% in cow's milk. Though the proteins of both these milks are of high nutritional value, that of cow's milk is of less value than that of human milk, possibly as much as 20% less."

[1] That portion of the study conducted at Washington University School of Medicine was aided by a grant from the Commonwealth Fund.

Some divergence from this view is expressed by Gordon, Levine, Wheatley and Marples ('37) who found that premature babies fed high but equal levels of protein from cow's and mother's milks retained equal amounts of nitrogen. Harrison ('36) fed a low nitrogen intake as cow's milk to normal infants and reported adequate nitrogen retention.

With the advent of parenteral alimentation and the use of casein as one of the principal sources of protein for hydrolysis, it becomes particularly necessary to know to what extent casein is deficient for human nutrition. The physical problems of parenteral alimentation properly emphasize the importance of choosing that nitrogen source most efficient for retention.

The results of animal studies on the addition of cystine to casein have never been confirmed in human beings. The only report on the nutritional value of added cystine for man is that of Pittman ('32) who added 2% of cystine to navy beans and found a slightly improved nitrogen retention. In the present study, rats, dogs, and human subjects have been employed in determining the value of adding cystine or methionine to a casein hydrolysate.

EXPERIMENTAL

Studies on dogs

In order to simulate the clinical conditions under which the hydrolysates would be used parenterally, 2 dogs were depleted in weight and protein stores by long feeding of the Weech-Goettsch low-protein diet ('38) to which was added a daily supplement of vitamins and a small amount of commercial dog food to supply in total 0.04 gm of nitrogen and 50 cal. per kilo. At the end of the depletion period of 12 weeks, the first dog had lost 128 gm of nitrogen, 1.25 kg in weight, and had a total plasma protein of 5.6 and albumin 3.8 gm % and a hematocrit reading of 48. Toward the end of the subsequent experimental periods a portion of the food was not eaten and the caloric intake was inadequate (see table 1).

TABLE 1

The effect of adding individual amino acids to casein hydrolysate given intravenously. Dog 68; mongrel male.

PERIOD (7 DAYS)	SUPPLEMENT [1]	DAILY CALORIC INTAKE PER KG	DAILY N INTAKE GM/DAY		TOTAL NITROGEN EXCRETION IN GM		AVERAGE DAILY N BALANCE GM	WEIGHT KG	PLASMA PROTEINS [3]		
			Food	Intravenous	Urine	Feces			Total gm %	Albumin gm %	Hematocrit
11	None	50.0	0.82		10.71	3.96	—1.28	20.4			
12	None	50.2	0.82		10.26	4.38	—1.27	20.5	5.7	3.8	58
13	Hydrolysate	51.4	0.82	1.16	12.03	3.85	—0.30	20.4	6.5	3.1	60
14	H + 5% VUK [2]	51.6	0.83	1.16	11.47	4.59	—0.31	20.9	6.3	3.2	56
15	H + 5% dl-threonine	51.0	0.83	1.16	11.42	4.32	—0.26	20.8	5.7	2.5	58
16	H + 5% dl-valine	51.7	0.84	1.16	10.37	4.66	—0.19	21.1	5.2	2.5	50
17	H + 5% dl-leucine	51.1	0.84	1.16	10.87	3.71	—0.09	21.1	5.7	2.6	53
18	H + 5% dl-isoleucine	51.4	0.85	1.16	10.62	4.61	—0.17	21.3	5.5	2.6	51
19	H + 5% l(+) lysine HCl	51.5	0.86	1.16	10.99	4.03	—0.13	21.4	5.3	2.7	53
20	H + 5% l(—) tryptophane	51.5	0.87	1.16	11.70	4.62	—0.31	21.7	5.5	2.5	52
21	H + 5% dl-phenylalanine	51.2	0.86	1.16	11.69	4.08	—0.24	21.5		2.5	54
22	H + 5% dl-methionine	48.8	0.84	1.16	7.43	4.69	+ 0.27	21.9	5.4	2.6	54
23	H + 5% l(+) histidine HCl	35.1	0.61	1.16	9.03	3.27	+ 0.01	22.0	5.9	2.9	52
24	H + 5% l(+) arginine HCl	33.0	0.58	1.16	10.30	4.05	—0.31	21.4	6.9	3.3	65
25	Hydrolysate	31.0	0.55	1.16	10.51	4.11	—0.38	21.4	5.8	2.9	56
26	H + 5% dl-methionine	43.7	0.74	1.16	7.19	3.25	+ 0.40	21.1	6.1	3.0	55
27	Hydrolysate	36.3	0.63	1.16	9.87	4.58	—0.28	21.2	5.5	2.9	56
28	H + 5% dl-leucine	36.7	0.69	1.16	10.63	3.56	—0.19	21.4	5.6	2.7	57
29	H + 5% dl-valine	34.3	0.59	1.16	9.90	3.57	—0.18	21.2	5.4	2.5	57
30	Commercial dog food	26.9	1.58		9.96	4.82	—0.53	21.1	5.5	2.6	54
31	H + 5% l(+) lysine HCl	32.2	0.54	1.16	10.23	3.48	—0.26	20.9	5.7	2.6	57
32	H + 5% l(+) histidine HCl	30.3	0.51	1.16	9.68	3.13	—0.15	20.5	5.7	2.8	56

[1] The supplement replaced 5% of the hydrolysate nitrogen.

[2] Mixture of essential amino acids devised by Madden et al., J. Exp. Med., *82*: 77.

[3] The values obtained at the end of the indicated period are given.

A 10% solution of the casein hydrolysate [2] sterilized by Seitz filtration, in an amount calculated to equal the daily nitrogen loss during the last few depletion periods was infused intraveneously at a constant rate of 1.33 mg N/kg/min. once daily for 7 days. During subsequent periods 5% of the hydrolysate nitrogen was substituted by an equal amount of nitrogen from 1 of the essential amino acids. Each test mixture was given for 7 days. The tabulated results are given in table 1. The last 2 weeks of depletion are included in the table for comparison.

The intravenous injection of 1.16 gm nitrogen as the hydrolysate did not lead to a positive balance, but spared about 0.97 gm of body nitrogen (Period 13). The substitution of a small amount of all 10 mixed essential amino acids did not improve retention (Period 14). Single amino acids seemed to exert a slight improvement but the only important change was caused by methionine (Periods 22 and 26). These 2 periods with added methionine gave a net nitrogen *retention* of 0.33 gm daily, which is to be compared with the 3 periods on the hydrolysate alone (Periods 13, 25, 27), which gave an average daily *loss* of 0.32 gm nitrogen. The nitrogen balance when valine, leucine, lysine and histidine were added was suggestive of improvement (Periods 16, 17, 19 and 23), but this was not confirmed on repetition (Periods 28, 29, 31 and 32).

The validity of the last few periods in this series is in question since the dog progressively refused to eat, with consequent drop in caloric intake. In Period 30, the 1.16 gm of nitrogen that had been given intravenously were fed in the form of commercial dog food, but a large amount of the carrot diet was refused.

The results with methionine were extended and confirmed in a second dog kept on the protein-poor diet for 11 weeks (nitrogen intake 0.04 gm per kg; caloric intake, 50 per kg), during which time he lost 6 kg in weight. Nitrogen loss was deter-

[2] All casein hydrolysate used in this study was prepared by pancreatic digestion in a manner previously described (Mueller, Kemmerer, Cox and Barnes, '40). The preparation is known under the trade name of Amigen.

TABLE 2

Effect of supplemental methionine on nitrogen retention when an enzymic casein hydrolysate is given orally, intravenously and subcutaneously.

Dog 103; male pointer.

PERIOD (7 DAYS)	SUPPLEMENT	DAILY CALORIC INTAKE PER KG	NITROGEN INTAKE GM/DAY		NITROGEN EXCRETION GM		AVERAGE DAILY N BALANCE GM	WEIGHT KG	PLASMA PROTEINS		
			Food	Supplement	Urine	Feces			Total gm %	Albumin gm %	Hemato-crit
10	None	50.0	0.61		12.90	5.56	—2.03	15.2	5.6	1.6	45
11	None	50.3	0.60		11.63	5.32	—1.85	14.9	5.3	1.5	41
12	Hydrolysate, i.v.	54.1	0.60	1.81	14.93	3.97	—0.29	14.9	5.7	1.6	42
13	Hydrolysate, i.v.	53.7	0.59	1.90	14.37	4.87	—0.26	14.9	5.3	1.8	39
14	Hydrolysate + Methionine, i.v.	53.4	0.60	1.90	12.46	3.51	+0.22	15.0	5.9	1.9	37
15	Hydrolysate, i.v.	53.7	0.60	1.90	14.67	4.30	—0.21	15.1	5.7	1.7	37
16	Hydrolysate + Methionine, i.v.	53.6	0.59	1.90	11.07	4.29	+0.29	14.7	5.7	1.8	36
17	Hydrolysate, i.v.	54.2	0.60	1.90	11.92	4.27	+0.19	15.1	5.5	1.6	37
18	Hydrolysate, i.v.	54.7	0.60	1.90	13.53	3.58	+0.06	14.9	5.8	2.0	41
19	Hydrolysate + Methionine, i.v.	53.5	0.59	1.90	9.62	3.62	+0.60	14.7	6.0	2.2	42
20	Hydrolysate, oral	54.3	0.60	1.90	11.02	3.44	+0.44	14.7	5.6	2.1	44
21	Hydrolysate + Methionine, oral	54.2	0.60	1.90	6.98	3.03	+0.93	15.0	5.7	2.8	44
22	Hydrolysate, oral	53.3	0.60	1.90	8.67	3.57	+0.75	15.1	5.3	2.3	44
23	Hydrolysate, oral	54.9	0.61	1.90	12.34	4.18	+0.15	15.0	5.5	2.3	45
24	Hydrolysate, oral	54.9	0.60	1.90	13.95	4.04	—0.07	14.9	5.7	2.3	48
25	Hydrolysate, s.c.	53.5	0.59	1.90	13.96	3.64	—0.03	14.7	5.4	2.4	
26	Hydrolysate + Methionine, s.c.	53.9	0.59	1.90	8.18	2.42	+0.98	14.7	5.9	2.4	44
27	Hydrolysate, i.v.	54.6	0.60	1.90	10.16	4.08	+0.47	15.1	5.8	2.7	46

mined for the last 5 weeks of depletion and averaged 1.90 gm daily. Intravenous supplementation with this amount of hydrolysate nitrogen (Periods 12 and 13) was followed by certain periods in which 5% of the hydrolysate nitrogen was replaced with an equal amount of methionine nitrogen. The data are given in table 2. It will be noticed that 1.90 gm hydrolysate nitrogen spared 1.68 gm of body nitrogen (Periods 10, 11 and 13), but the substitution of methionine resulted in actual nitrogen retention (Period 14). The improved nitrogen retention with the methionine supplement persists whether the material is given intravenously (compare Periods 13, 14 and 15), orally (compare Periods 20, 21, 22, 23 and 24) or subcutaneously (Periods 25 and 26). The beneficial effect of

TABLE 3

Comparison of nitrogen balance after administering casein hydrolysate by different routes.

DOG 103	GM NITROGEN PER DAY	
Nitrogen source	Casein Hydrolysate	Casein Hydrolysate + Methionine
Intravenous (Periods 13, 14, 16, 19)	— 0.26	+ 0.37
Subcutaneous (Periods 25, 26)	— 0.03	+ 0.98
Oral (Periods 24, 21)	— 0.07	+ 0.93

methionine on nitrogen retention persists for at least 2 weeks following its withdrawal. This is best shown in Period 21 when the oral administration of the hydrolysate with methionine resulted in a retention of 0.93 gm daily, and when the methionine was omitted in 3 subsequent periods, the retention dropped progressively, + 0.75, + 0.15, — 0.07 gm daily.

The data permit a rough comparison of different routes of administration. As shown in table 3, the subcutaneous and oral routes give definitely higher retentions than the intravenous. When the hydrolysate was given without supplementation, intravenous infusion gave a nitrogen balance of — 0.26; subcutaneous + 0.98 (Period 26); and oral + 0.93 (Period 21) gm daily. This difference may be due in part to a

greater renal excretion of amino acids or peptides following intravenous infusion (Cox and Mueller, '46).

Studies on rats

Two hydrolysates of casein prepared in an identical manner except that 1 was digested with tryptic enzyme from pancreas (Mueller, Kemmerer, Cox and Barnes, '40) and the other with tryptic enzyme from the pyloric caeca of fish (Bondouy, 1899), were each fed to 10 young rats at a level of 1.2% nitrogen (equivalent to 7.5% protein). Additional groups were fed similarly except that 2½% [3] of hydrolysate nitrogen was replaced by an equal amount of cystine and of methionine nitrogen, so that the total nitrogen intake (1.2%) remained constant. Young albino rats weighing between 35 and 45 gm, and distributed as to sex and litter, were placed at weaning on each of the 6 experimental diets. The rats were housed in individual wire-bottomed cages and fed tap water and the diet ad libitum. The basal diet consisted of lard 9.0, salt mixture 4.0, cod liver oil 2.0, wheat germ oil 1.0, brewers' yeast 2.0, thiamine hydrochloride 0.0006, and riboflavin 0.0002%, and enough hydrolysate added to supply 1.2% nitrogen and dextrin sufficient to make 100 gm.

The average weight gains are given in table 4. As expected from the early work of Osborne and Mendel ('15) and Womack, Kemmerer and Rose ('37), both supplemented diets resulted in increased weight gain. Methionine consistently gave greater gains than cystine. In order to determine that the gain in weight was not due to water or fat retention, the rats were killed at 8 weeks, the gastrointestinal tract removed with as little loss of blood as possible, and the individual rats placed in pint jars and autoclaved under steam pressure. They were ground and analyzed individually for total

[3] This level was chosen after preliminary experiments in which 1.25, 2.5, 5.0, 7.5 and 10% of methionine nitrogen had been substituted for an equal amount of hydrolysate nitrogen. Growth on the 2 lowest levels was equal, and greater than on the other 3.

TABLE 4

Average gain in weight and per gram nitrogen ingested of 10 rats fed 1.2% nitrogen for 8 weeks as an enzymic hydrolysate of casein with supplements of cystine and methionine (2½% of the hydrolysate nitrogen).

HYDROLYSATE EMPLOYED	AVERAGE GAIN IN WEIGHT		GAIN PER GM N INGESTED	
	A	P	A	P
	gm	*gm*	*gm*	*gm*
Hydrolysate without supplement	112	91	15.7	14.6
Hydrolysate with cystine	131	115	18.9	17.8
Hydrolysate with methionine	154	129	20.6	19.0
Gain due to cystine	19	24	3.2	3.2
Gain due to methionine	42	38	4.9	4.4

TABLE 5

Average composition of rats and calculated composition of the gain as total solids, protein, ash and fat.

	% COMPOSITION				COMPOSITION OF THE GAIN IN GM PER AVERAGE RAT			
Substance fed	Solids	Nitrogen	Ash	Lipids	Solids	N × 6.25	Ash	Lipids
Hydrolysate A	40.28	3.23	3.85	16.77	45.1	22.6	4.31	18.8
Hydrolysate + Cystine	39.09	3.36	3.98	14.50	51.2	27.5	5.21	19.0
Hydrolysate + Methionine	39.10	3.31	3.76	15.19	60.2	31.9	5.79	23.4
Hydrolysate P	39.08	3.34	4.35	14.34	35.6	19.0	3.96	13.1
Hydrolysate + Cystine	39.34	3.28	4.05	15.08	45.2	23.6	4.66	17.3
Hydrolysate + Methionine	40.63	3.28	3.74	16.36	52.4	26.4	4.82	21.1

solids, ash, protein and fat, using standard analytic methods.[4] The results are given in table 5. There is no significant difference in the per cent total solids, nitrogen, ash or lipids when the hydrolysate group is compared with the groups receiving additional sulfur-containing amino acids. The actual

[4] We wish to express our appreciation to Mr. Charles Wesselman for these analyses.

composition of the gain shows that there was an absolute increase in body protein, ash, solids and fat, which paralleled the increase in total weight. If the weight of the rat is plotted against the quantitative gain in each of these 4 categories, the values fall along a straight line, indicating that the gain in weight of the methionine and cystine rats was a real gain and not due to retention of water or to the deposition of excessive fat.

Observations on human beings

Patients. Four groups of human subjects have been used in studying the effect of added methionine. The first group consisted of 3 patients with pyloric obstruction who were being prepared for operation. For the period of this experiment they took no food of any kind by mouth and all gastric secretions were constantly drained by tube. The patients were all emaciated, having lost from 20 to 40 pounds in body weight and were obviously in need of protein. As the sole source of food, 2 liters of Amigen 5% in 5% Dextrose Solution were given each patient daily for 5 days and the amount of urinary nitrogen and that lost by gastric lavage determined daily. No fecal samples were excreted since no food was ingested. During a second consecutive 5-day period, 3.2 gm of methionine replaced an equal amount of hydrolysate, the same amount of total nitrogen and calories being injected daily as in the 5 previous days. As shown in table 6, 1 of the 3 subjects was in nitrogen balance at this level of nitrogen and caloric alimentation while the other 2 were in slightly negative balance. The addition of methionine to the solution of the hydrolysate during the second 5-day period actually resulted in a worsened balance in 2 of the patients and an improvement in the other. From this it was clear that the methionine had had no beneficial effect on nitrogen retention.

Normal infants. We were somewhat puzzled at this negative finding for methionine and thought that it might possibly have been due to an inadequate caloric intake, or to some abnormality of metabolism related to the existing gastrointestinal

disease. With the idea that the beneficial effects of added methionine, if any, might be more clearly demonstrated in the growing organism, nitrogen balance experiments were performed on 2 normal, white, full-term male infants. They were maintained on a standard metabolism frame adapted for

TABLE 6

Nitrogen balance of 3 male patients with pyloric obstruction being prepared for gastric resection. Two liters of Amigen 5% in 5% dextrose solution intravenously with and without added methionine (a total of 12.7 gm nitrogen) were given each patient daily.

Day	PATIENT F: 63 years, wt. 45 kg			PATIENT H 66 years, wt. 51 kg			PATIENT M. 62 years, wt. 67 kg		
	Urinary N	Gastric contents N	N balance	Urinary N	Gastric contents N	N balance	Urinary N	Gastric contents N	N balance
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
	Hydrolysate infusion without supplement								
1	8.20	0.68	+ 3.8	11.48	0.44	+ 0.8	13.65	0.50	— 1.5
2	8.27	1.63	+ 2.8	14.28	0.12	— 1.7	13.89	0.52	— 1.7
3	9.80	1.29	+ 1.6	11.48	0.19	+ 1.0	16.42	0.29	— 4.0
4	8.93	1.15	+ 2.6	15.43	0.12	— 2.9	15.39	0.26	— 3.0
5	9.15	1.16	+ 2.4	11.96	0.19	+ 0.5	13.94	0.32	— 1.6
Average			+ 2.6			— 0.5			— 2.4
	With methionine; 3.2 gm daily replacing an equal amount of hydrolysate								
6	12.31	1.15	— 0.8	15.07	1.03	— 3.4	12.50	0.69	— 0.5
7	9.98	0.98	+ 1.6	14.08	0.09	— 1.5	13.06	inc.	— 0.4
8	10.62	0.90	+ 1.2	14.14	inc.	— 1.4	15.03	0.15	— 2.5
9	11.03	0.86	+ 0.8	15.45	0.06	— 2.8	14.69	0.11	— 2.1
10	inc.	inc.		13.08	0.08	— 0.5	14.01	0.07	— 1.4
Average			+ 0.7			— 1.9			— 1.4

the collection of urine and feces. The diet periods began on each Saturday morning and during the first 2 days the urine and feces were not collected in order to allow adjustments to the diet and rest from continued restraint. The infants were placed on metabolism frames at the end of these 2 days and daily collections continued for 5 days. The diet was composed

of casein hydrolysate, 3.5 gm; Laboratory Product no. 217,[5] 18 gm; dried brewers' yeast, 1 gm dissolved in sufficient water to make 100 ml. One cubic centimeter of the solution supplied 1 calorie.

The diet was given in 5 feedings daily at 100 cal. per kilo. body weight and was supplemented with 50 mg of ascorbic acid and 15 drops of Oleum Percomorphum. The daily intake was calculated from the food consumption record, and from the

TABLE 7

Nitrogen balance in infants, effect of adding methionine to a casein hydrolysate.

	PATIENT P. D., 7381 GM WEIGHT		PATIENT D. R., 7073 GM WEIGHT	
Nitrogen source	Hydrolysate	H + Methionine	Hydrolysate	H + Methionine
Period, 5 days each	1	2	1	2
Total Nitrogen intake, gm	5.03	5.20	4.70	4.84
Nitrogen absorption, mg/kg/day	585	603	639	671
Nitrogen retention, mg/kg/day	182	182	309	297
Nitrogen retention, gm/day	1.40	1.45	2.19	2.14
Gain in weight, gm/day	36.0	32.4	9.7	28.7

analysis of a composite sample of the 7 daily formulas. During the second period 3% of methionine was added to the casein hydrolysate and all other quantities maintained constant.

Nitrogen absorption was calculated by subtracting fecal nitrogen from the intake; and retention by subtracting the urinary nitrogen from that absorbed. It is evident from table 7 that the net retention, expressed in grams per day, was identical in the supplemented and unsupplemented periods.

[5] Consisting of Dextri-Maltose no. 2, 54.6; milk fat, 23.9; arrowroot starch, 13.0, calcium gluconate, 4.4; KH_2PO_4, 1.8; $Ca(OH)_2$, 1.1; K_2HPO_4, 0.6; KCl, 0.5; Mg O, 0.1 and $FeSO_4$, 0.02 gm.

The addition of methionine did not improve nitrogen retention. This second failure of methionine to improve nitrogen retention even in growing babies was most surprising. However, the cystine deficiency of casein is only demonstrable in the rat at low levels of protein intake (Osborne and Mendel, '15); and these balance studies on babies were made at a nitrogen intake equivalent to 4 to 5 gm of protein per kg body weight. This intake level may possibly have been sufficiently high to mask any inadequacy of the casein hydrolysate.

Normal adults at a maintenance level of nitrogen. In view of these 2 negative results for added methionine in the human subjects, we felt that further studies with minimal intakes might give results more in accord with those found in rats and dogs. Two groups of adult volunteers,[6] all laboratory workers, collaborated in the studies. One group of 6 subjects was studied at a maintenance level, and the other group of 4 subjects was on a sub-maintenance level. All subjects were completely normal on physical examination, including the usual blood and urine analyses. In the maintenance study 3 men and 3 women were placed for 7 days on a regulated diet of natural foods supplying 40 cal. and 0.11 gm of nitrogen per kg daily. The protein-low diet of Mueller, Fickas and Cox ('43) was then substituted but the nitrogen intake level was kept constant at 0.11 gm per kg by the addition of casein hydrolysate. The low protein diet supplied 0.01 gm of nitrogen per kg or 9% of the total nitrogen intake. Daily urinary and fecal nitrogen determinations were made. It is evident from figure 1 that the hydrolysate was as effective in maintaining nitrogen balance (Periods 3 and 4) as regular food protein (Period 2).

The hydrolysate was then removed from the diet for a period of 4 days, with marked nitrogen loss (Period 5). When restored at the same level as previously, there was nitrogen

[6] These volunteers were: Misses Edna Fisher, Kathryn Drone, Theodora Block, Messrs. William Koch, Edward Bonham, Donald Schneider, K. S. Kemmerer, R. W. Barton, A. J. Mueller and W. M. Cox, Jr. We want to express our appreciation of their help.

retention (Period 6). During the last 4-day period, 2½% of the hydrolysate nitrogen was replaced by an equal amount of cystine nitrogen (Period 7) but without any increase in nitrogen retention. This finding is again in contradistinction to the animal findings. In trying to rationalize why there should be such a marked difference in the effect of added methionine

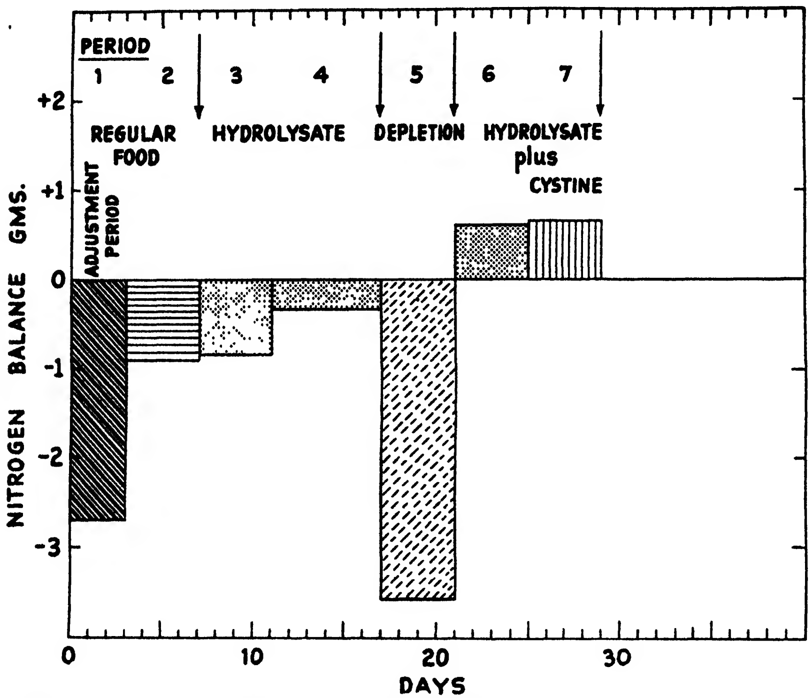


Fig. 1 Average nitrogen balances of 6 adult human subjects maintained on a diet supplying 0.11 gm of nitrogen and 40 cal. per kg body weight daily. During the periods when the enzymic casein hydrolysate was given, the protein-low diet of Mueller, Fickas and Cox ('43) was given. Daily balances were determined and averaged as 3, 4 or 6-day periods.

in 2 species, it was evident that (1) the dogs were protein-depleted whereas the humans were merely on a maintenance diet without any significant depletion and (2) the nitrogen intake of the human subjects was the same per kg body weight, whereas the intake of the dogs was adjusted to their endogenous loss.

Normal adults depleted of protein. As a final test for the value of added methionine in man, it was decided to deplete our 4 human volunteers of their protein stores in a manner similar to that employed with the dogs and then to supplement the diet by that amount of nitrogen which each was losing daily. Four normal men ingested the protein-low diet previously described (Mueller, Fickas and Cox, '43) for a period of 3 weeks. Nitrogen analyses on the foodstuffs were made and the actual intake level established at 0.01 gm of nitrogen per kg. The caloric intake was maintained at 30 per kg throughout the entire experiment. This is 1.4 times the average basal caloric requirement when calculated on the basis of surface area (height-weight formula of DuBois and DuBois, '16). Other investigators, particularly Martin and Robison ('22) have emphasized the difficulty of ingesting protein-free foods for protracted periods. While adjustment to the diet of starch-milkfat crackers, milk fat, vegetable oils, sugar and small amounts of low-nitrogen vegetables and fruits, as lettuce, cabbage, peaches and apricots was not easy, the principal objection was that of monotony. All subjects continued their usual laboratory and home-gardening activities, but about 1 day in 5 each was depressed and felt that he couldn't continue the undertaking. Our early observations of lassitude after 4 days depletion (Mueller, Fickas and Cox, '43) were probably due to such temporary periods of adjustment to the diet, which usually disappeared within 24 hours with a return of a normal reaction towards activity and duties. Urinary and fecal nitrogen was determined daily, and the nitrogen in coffee was counted as part of the total nitrogen intake.

As noted above, all subjects were proved normal by the usual clinical and chemical examinations and the measurements of most interest for the purpose of this study — weight, total serum protein and serum albumin, and hematocrit — are given in table 8. During depletion, similar measurements were made at weekly intervals and at the end of the experimental period (table 8). It will be seen from these data that the weight loss was between 3.2 and 5.2 kg. The nitrogen loss during the

whole experiment multiplied by the customary 30 to convert it to a weight equivalent gave an indicated weight loss of from 2.2 to 3.2 kg in the 4 subjects. This calculated weight loss is not greatly different from the actual weight loss, indicating that the caloric intake of 30 per kg was adequate, although it should be noted that subject (A), performing the most work, lost the greatest weight. Other investigators (Martin and

TABLE 8

Weight, serum protein, serum albumin and hematocrit values during protein depletion of 4 subjects for 21 days, and after 10 days of minimal hydrolysate supplementation.

SUBJECT	A	B	C	D
	kg	*kg*	*kg*	*kg*
Weight, initial	91.2	72.1	84.7	82.1
1 wk. depletion, loss	—1.8	—0.9	—0.2	—1.8
2 wks. depletion, loss	—0.7	—2.4	—1.6	—0.7
3 wks. depletion, loss	—2.2	—0.2	—0.3	—0.8
End of supplementation	—0.5	—0.2	—1.2	+0.1
Total weight loss	—5.2	—3.7	—3.3	—3.2
Total nitrogen loss, gm	106.7	89.4	71.4	89.3
N. loss × 30, kg	3.2	2.7	2.2	2.7
	gm %	*gm %*	*gm %*	*gm %*
Serum albumin, initial	4.66	4.91	4.91	4.67
1 wk. depletion	4.35	4.60	4.62	4.32
2 wks. depletion	4.31	4.81	4.17	4.10
3 wks. depletion	4.18	4.37	4.39	4.23
Change during depletion	—.48	—.54	—.52	—.44
End of supplementation	3.99	4.50	4.43	4.37
	gm %	*gm %*	*gm %*	*gm %*
Serum protein, initial	6.87	7.09	7.30	7.43
1 wk. depletion	7.06	6.72	6.84	6.46
2 wks. depletion	6.54	7.05	6.26	6.10
3 wks. depletion	6.25	6.56	6.80	6.19
Change during depletion	—.62	—.53	—.50	—1.24
End of supplementation	6.39	6.79	6.59	6.46
	% c.v.	*% c.v.*	*% c.v.*	*% c.v.*
Hematocrit, initial	45.1	48.5	49.9	46.3
1 wk. depletion	46.0	47.3	51.4	48.0
2 wks. depletion	47.2	47.0	50.3	44.4
3 wks. depletion	49.5	43.2	55.9	44.1
End of supplementation	47.0	40.6	51.8	42.6

Robison, '22) have employed higher caloric intakes, but a large part of their difficulty with the ingestion of the diets is probably due to this high food intake.

The total serum protein and serum albumin values declined progressively during depletion, in a manner and degree comparable to that previously observed in dogs (Cox and Mueller, '44). There was no significant regeneration of protein during supplementation, since the level of nitrogen given was inadequate to attain nitrogen balance. There was but little change in the hematocrit values during the experiment, so that the fall in percentage albumin may be considered as valid.

TABLE 9

Summarized data on 4 subjects for the last 6 days of a 21-day protein depletion period followed by two 4-day periods supplemented with casein hydrolysate with and without added methionine.

SUBJECT	A	B	C	D
Food N, gm/day, last 6 days	0.91	0.72	0.85	0.82
Urinary N, gm/day, last 6 days	3.44	2.88	2.33	2.68
Fecal N, gm/day, last 6 days	1.03	0.40	0.79	0.70
Net nitrogen loss during last 6 days of depletion	—3.56	—2.56	—2.27	—2.56
Nitrogen balance during hydrolysate supplementation	—0.97	—0.96	—0.76	—1.36
Nitrogen balance during hydrolysate and methionine supplementation	—0.99	—1.06	—0.91	—1.29

At the end of 21 days depletion, hydrolysate was added to the diet. The amount given was equal to the average amount of nitrogen lost by each subject during the last 6 days of the 21-day period and was administered for the next 4 consecutive days as the casein hydrolysate. As shown in table 9 (line 4), the net nitrogen loss was 3.56, 2.56, 2.27 and 2.56 gm daily for the 4 subjects. These values are in very close agreement with the endogenous loss reported by others under similar protein depletion. For example, Martin and Robison ('22) report an endogenous loss of 3.17 and 2.89 gm nitrogen per

day. The average amount of hydrolysate required to furnish the required amount of nitrogen was only 22.3 gm daily. As shown in line 5 (table 9), balance was not achieved although the nitrogen loss was considerably reduced by the supplement. The average loss of — 2.74 gm was reduced to — 1.01 gm daily. A second period followed in which 2½% of the hydrolysate nitrogen was substituted by an equal amount of nitrogen

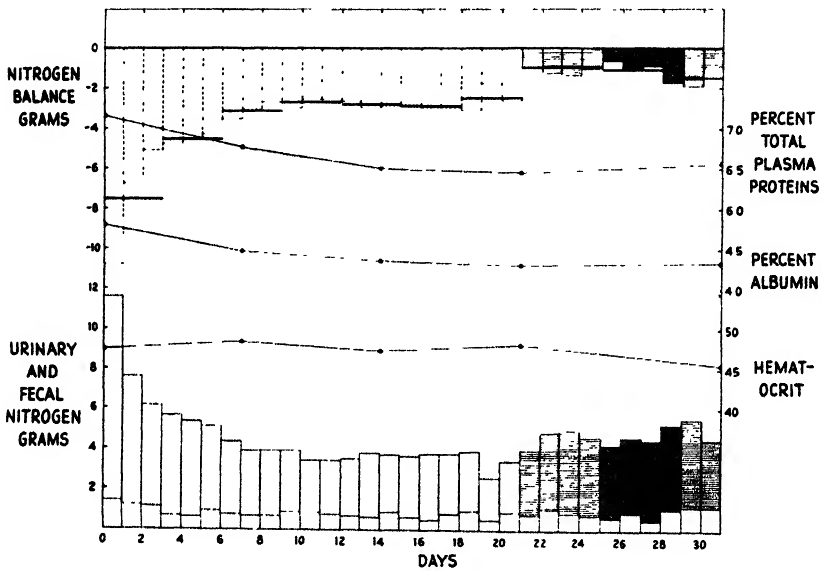


Fig. 2 Nitrogen balance, urinary and fecal nitrogen and blood values of 4 human adult males receiving a protein-low diet (0.01 gm N per kg per day) and 30 cal. per kg body weight for a period of 21 days. Supplementation with an enzymic casein hydrolysate for 4 days (horizontal lines, 22 to 25th day) and with the same hydrolysate and a methionine supplement (diagonal lines, 26 to 29th day) for 4 days followed depletion. The values for each day are given and the heavy lines indicate the average nitrogen balance for 3- or for 4-day periods.

as methionine. The average weighed amount of methionine was 0.763 gm daily for each of the 4 subjects. As shown in the last line of table 9, and in figure 2, the added methionine led to no more reduction in nitrogen loss than the hydrolysate alone, since the average nitrogen balance for the 4 subjects was — 1.06 gm daily, as compared with — 1.01 for the hydrolysate period immediately preceding.

This finding demonstrated to us that the addition of methionine to a casein hydrolysate did not increase the efficiency of nitrogen retention in humans.

DISCUSSION

A comparison of the nitrogen retention of a casein hydrolysate with and without added methionine in rats, dogs and man has clearly shown a striking species difference. The addition of methionine increased the rate of growth in rats and the magnitude of nitrogen retention in dogs. In man, however, it was without effect on nitrogen retention even though observations were made on 4 different groups: (1) depleted surgical patients fed intravenously (2) normal adults fed at a maintenance nitrogen level (3) normal adults who had been protein depleted for 3 weeks and were then fed only an amount of nitrogen equal to their endogenous loss, and (4) normal infants. In no one of these 4 groups did the addition of extra amounts of the essential sulphur-containing amino acid increase nitrogen retention.

An explanation for this difference does not seem difficult, based on the fact that the rat and dog are covered with hair, and that man is not. Since hair contains large amounts of cystine (Block and Bolling, '45) it is reasonable to suppose that the requirement of the rat and dog for this amino acid (or methionine) is considerably greater than that of man.

Whether this explanation is valid or not, the implications of the finding are large. We employed an hydrolysate of casein but it seems reasonable to interpret the data as though casein itself were employed, inasmuch as the growth of rats receiving the hydrolysate was equal to that of those receiving casein (Mueller, Kemmerer, Cox and Barnes, '40). Moreover, supplementation of the hydrolysate with cystine and methionine gave the same improvement in growth as when casein (unpublished studies) was similarly supplemented.

The finding of Osborne and Mendel ('15) that casein was deficient in cystine was based on rat observation. It was promptly (and at that time, perhaps properly) used in ex-

plaining the contemporary observation that infants failed to develop as well on artificial feedings as on human breast milk (Holt, '16). Thus, it was argued that since the protein of cow's milk consists preponderantly of casein with a low cystine content, whereas breast milk contains principally lactalbumin with a high content of sulfur-containing amino acids, it is logical to attribute the inferior results from cow's milk feedings to this fact. With improvement in the bacteriological quality of cow's milk, the supposed nutritional differences between casein and lactalbumin became much less necessary or evident. Gordon, Levine, Wheatley and Marples ('37) studied infants fed similar amounts of nitrogen as cow's milk and as breast milk and could find no significant difference in their nitrogen retention. Harrison ('36) reported that when the intake of cow's milk protein was quantitatively reduced to that ingested in breast milk, good nitrogen retention was observed. These earlier observations support the present finding.

On the basis of the work of Osborne and Mendel ('15) it would have been logical to add cystine to cow's milk to increase its nutritive value. To our knowledge, this experiment was not done. Our findings indicate that neither the addition of cystine or methionine should have increased the nutritive value of the milk for babies, even though it might have done so for the rat. That there is no difference in the nutritive value of casein and lactalbumin for man based on a difference in sulfur-containing amino acid content is clear from the present findings, but to establish the relative nutritive value of these 2 proteins, casein and lactalbumin, *for man*, it will be necessary to compare carefully their growth-promoting qualities and their effectiveness for nitrogen retention *in man*. Experimental work with rats will not suffice. The assumed nutritive difference between casein and lactalbumin has been established *for the rat and dog*, but not for man. Our wofk serves as another illustration of the point that the results with 1 species cannot always be applied to other species.

SUMMARY

1. The addition of cystine or methionine to a casein hydrolysate given as the sole source of nitrogenous food increased the rate of growth in rats.

2. When the casein hydrolysate was given by vein to dogs, the addition of methionine increased nitrogen retention, whereas the addition singly of the other 9 essential amino acids had no such effect.

3. In 4 groups of human subjects, the addition of methionine to a casein hydrolysate given as the sole source of nitrogenous food, did not increase nitrogen retention. This was true in surgical patients to whom the material was given intravenously, in infants fed a luxury level of nitrogen, and in well adults fed a maintenance nitrogen level.

4. When 4 human subjects were protein-depleted for 21 days, the subsequent administration of minimal amounts of an enzymic casein hydrolysate, with and without extra methionine, gave identical nitrogen retention.

5. These data are interpreted to mean that there is a species difference in the requirement for sulfur-containing amino acids, and that the human requirement for them is less than the requirement of the rat and dog, due presumably to a greater need for cystine in the building of hair.

6. The generally recognized nutritive difference between casein and lactalbumin is valid for the rat and for the dog, but not for man.

LITERATURE CITED

BLOCK, R. J., AND D. BOLLING 1945 The Amino Acid Composition of Protein and Foods. Charles C. Thomas, Springfield, p. 302.

BONDOUY, TH. 1899 Du role des tubes pyloriques, dans la digestion chez les teleosteens. Arch. zool. exp. gen. 3rd series. V 7 (V. 27): 419.

COX, WARREN M., JR., AND A. J. MUELLER 1944 Serum albumin regeneration as effected by intravenously and orally administered protein hydrolysates. J. Clin. Invest., *23:* 875.

——— 1946 The relative efficiency of different forms of intravenously administered nitrogen on nitrogen balance and amino acid excretion. J. Nutrition, *31:* 581.

DuBois, D., and E. F. DuBois 1916 Clinical calorimetry. X. A formula to estimate the approximate surface area if height and weight be known. Arch. Int. Med., *17:* 863.

Gordon, H. H., S. Z. Levine, M. A. Wheatley and E. Marples 1937 Respiratory metabolism in infancy and childhood. XX. The nitrogen metabolism in premature infants — Comparative studies of human milk and cow's milk. Am. J. Dis. Child., *54:* 1030.

Harrison, H. E. 1936 The retentions of nitrogen, calcium and phosphorus of infants fed sweetened condensed milk. J. Pediat., *8:* 415.

Holt, L. E. 1916 The digestibility of the proteins of milk and their role in infant nutrition. Arch. Ped., *33:* 13.

Marriott, W. McK., and P. C. Jeans 1941 Infant Nutrition, 3rd Ed., C. V. Mosby, St. Louis, p. 49.

Martin, C. J., and R. Robison 1922 The minimum nitrogen expenditure of man and the biological value of various proteins for human nutrition. Biochem. J., *16:* 407.

Mueller, A. J., D. Fickas and W. M. Cox, Jr. 1943 Minimum maintenance requirement of an enzymic casein hydrolysate. Bull. Johns Hopkins Hosp., *72:* 110.

Mueller, A. J., K. S. Kemmerer, W. M. Cox, Jr. and S. T. Barnes 1940 The effect of casein and a casein digest on growth and serum protein regeneration. J. Biol. Chem., *134:* 573.

Osborne, T. B., and L. B. Mendel 1915 The comparative nutritive value of certain proteins in growth, and the problems of the protein minimum. J. Biol. Chem., *20:* 351.

Pittman, M. S. 1932 The utilization by human subjects of the nitrogen, calcium and phosphorus of the navy bean (Phaseolus vulgaris) with and without a supplement of cystine. J. Nutrition, *5:* 277.

Unpublished studies, conducted simultaneously with the growth observations in rats reported in this paper.

Weech, A. A., and E. Goettsch 1938 Dietary protein and the regeneration of serum albumin. I. Method of assay and discussion of principles. Bull. Johns Hopkins Hosp., *63:* 154.

Womack, M., K. S. Kemmerer and W. C. Rose 1937 Relation of cystine and methionine to growth. J. Biol. Chem., *121:* 403.

THE CONJOINT EFFECTS OF VARIED INTAKES OF THIAMINE AND VITAMIN A ON RATS [1]

MARIE DYE, THELMA PORTER [2] AND EUNICE KELLY [3]

Department of Foods and Nutrition, School of Home Economics, Michigan State College, East Lansing

(Received for publication October 25, 1946)

This is a report of the fourth experiment in a series planned to show the effect of various dietary factors upon the utilization of vitamin A. In previous studies, it was found that increased caloric intake was responsible for greater weight gains than the unitage of vitamin A given, and that the percentage of fat and protein fed had no marked effect upon the utilization of vitamin A (Muelder and Kelly, '41, '42; Dye, Bateman and Porter, '45).

Few studies relating to the synergism of thiamine and vitamin A have been reported. In 1936, Randoin and Netter found that the young rat developed and matured rapidly in the presence of the B-vitamins whether or not vitamin A was present. It was not until the sixth week of their experimental low-vitamin A regime that the absence of vitamin A began to show its effect. Scheunert and co-workers ('37, '38) published papers on the effect of different quantities of vitamin A on the thiamine requirements and the effect of different quantities of thiamine on the vitamin A requirements of rats. Thiamine requirements of growing rats remained the same when the

[1] Authorized for publication as Journal Article no. 747 (N.S.) of the Michigan Agricultural Experiment Station.

[2] Present address: Department of Home Economics, University of Chicago, Chicago, Illinois.

[3] Present address: Agricultural Experiment Station, North Dakota Agricultural College, State College Station, Fargo, North Dakota.

vitamin A intake varied from 5 to 2000 units daily. Furthermore, altering the thiamine intake did not alter the quantity of vitamin A required for curative purposes. More recently, Mitalo ('42) found that, if thiamine and vitamin A were both lacking in the diet, the loss of vitamin A from the livers of the experimental animals was more rapid than when vitamin A alone was absent from the diet. It is difficult to interpret these findings because of variations in experimental procedure. It seemed of interest to set up an experiment to test further the conjoint effects of varied intakes of thiamine and vitamin A.

EXPERIMENTAL PROCEDURE

Depletion period

At weaning, triads of rats of the same sex, weight, and litter were placed upon a vitamin-A-depletion diet of the following percentage composition: casein (A and thiamine extracted) 18, irradiated corn-starch 73, fat 5, and Osborne and Mendel salt mixture 4. Supplements of 0.5 mg autoclaved yeast and 3 μg of thiamine as thiamine chloride were administered daily. Water was given ad libitum. Weekly food consumptions and weight records were kept during this time. The criteria used for depletion were a plateau in weight and signs of xerophthalmia.

Experimental period

At depletion, rats in 2 series, one having the food intake controlled to the smallest quantity eaten by any member of the triad, and the other receiving the diet ad libitum were placed on various levels of vitamin A and thiamine. The levels of vitamin A were 0, ½, 1½, or 3 I.U. per day, combined at each level with 0, 6, or 9 μg of thiamine per day. When 4 rats of the same sex, weight and litter were available the fourth rat was fed 18 μg of thiamine per day. The experimental period lasted for 6 weeks during which daily food consumptions and weekly weight records were kept. At the end of the experimental period, the surviving animals were killed with chloro-

form and gross autopsy findings and length of the animal were recorded.

The average findings on food consumption, weight changes and length of rat were subjected to tests for significant differences according to the "t" formula.

RESULTS AND DISCUSSION

Male and female triads of rats were depleted of vitamin A in an average of 6.5 weeks. Average weekly food consumptions at the time of reaching a growth plateau ranged from 31 to 33 gm, with average weights ranging from 91 to 95 gm. The criteria used to evaluate the effect of varying levels of thiamine in the diet upon the utilization of vitamin A were (1) food intake, (2) weight change, (3) length of the rat at the end of the experimental period, and (4) number of foci of keratinized epithelial tissues, called "abscesses." Table 1 gives a summary of the results from the animals receiving a controlled food intake, and table 2, the results from animals receiving the diet ad libitum. Some results of feeding 6 I.U. of vitamin A were obtained. However, only the triads at levels of 0, 1½ and 3 I.U. of vitamin A will be considered in the discussion because of equalization of numbers and sex in these groups.

The daily addition of 6 or 9 μg of thiamine to the diet, whether or not vitamin A was present significantly increased food intakes and favorably affected weight changes in all groups of animals. For the controlled feeding group (table 1) members of the triad surviving beyond the death of the rat receiving no thiamine were permitted to eat 3 gm of food daily for the remainder of the 6-week experimental period. From the table it may be seen that these increased food intakes resulted in corresponding increased weight gains at all levels of vitamin A. The average weekly food consumption for all animals receiving no thiamine in the controlled feeding group was 11.3 gm. A similar weekly food consumption average, 11.2 gm, was observed for all animals having access to the diet ad libitum (table 2) when no thiamine was present, thus demonstrating the direct effect of thiamine upon appetite.

TABLE 1

Controlled feeding group: Average weights, food consumption, and autopsy findings on animals on a 6-week experimental period.

VITAMIN LEVELS		NUMBER OF RATS	WEEKLY AVERAGE			GAIN PER CALORIE INGESTED	LENGTH OF RAT AT END OF EXPERIMENT [1]	"ABSCESSES" EVIDENT AT AUTOPSY [2]
Thiamine	Vitamin A		Food intake [1]	Calorie intake	Weight change [1]			
μg	*I.U.*		*gm*		*gm*	*gm*	*in.*	*no.*
0	0	6	14.4 ± 1.5	59.0	− 9.9 ± 1.3	−0.16	13.3 ± 0.0	3.2 ± 0.7
6	0	6	19.4 ± 0.8	79.5	− 3.4 ± 1.1	−0.04	12.5 ± 0.2	6.0 ± 0.8
9	0	6	20.3 ± 0.6	83.2	− 2.7 ± 0.8	−0.03	12.2 ± 0.5	4.7 ± 1.0
18	0	4	20.6 ± 0.5	84.5	− 3.2 ± 0.8	−0.04	12.7 ± 0.0	6.8 ± 0.8
0	½	6	8.8 ± 0.9	36.1	−13.7 ± 1.0	−0.32	12.1 ± 1.4	2.3 ± 0.5
6	½	6	20.9 ± 0.1	85.7	− 0.3 ± 0.6	−0.01	12.4 ± 0.4	1.5 ± 0.4
9	½	6	21.0 ± 0.1	86.1	− 0.4 ± 0.8	−0.01	12.6 ± 0.2	1.3 ± 0.7
18	½	2	20.9 ± 0.1	85.7	+ 2.8 ± 0.8	+0.03	12.5 ± 0.0	1.5 ± 0.4
0	1½	6	11.4 ± 0.9	46.7	− 7.8 ± 1.5	−0.17	12.1 ± 0.4	2.0 ± 0.6
6	1½	6	21.2 ± 0.4	86.9	+ 0.4 ± 0.9	+0.01	12.6 ± 0.3	0.3 ± 0.2
9	1½	6	21.3 ± 0.3	87.3	+ 1.4 ± 0.7	+0.02	12.4 ± 0.2	0.5 ± 0.3
18	1½	5	21.3 ± 0.4	87.3	+ 0.9 ± 0.6	+0.01	12.3 ± 0.1	0.6 ± 0.4
0	3	6	10.4 ± 1.0	42.6	−11.6 ± 1.0	−0.27	12.2 ± 0.3	1.7 ± 0.8
6	3	6	21.0 ± 0.7	86.1	+ 0.9 ± 0.7	+0.01	12.6 ± 0.0	0.7 ± 0.5
9	3	6	21.0 ± 0.9	86.1	+ 0.8 ± 0.7	+0.01	12.6 ± 0.1	0.2 ± 0.2
18	3	3	21.0 ± 0.0	86.1	+ 1.4 ± 0.9	+0.02	12.6 ± 0.3	0.7 ± 0.5

[1] Arithmetic means ± standard error.

[2] Areas that most frequently showed "abscesses" were middle ear, base of tongue, lymph glands in neck, salivary glands, and genito-urinary tract.

As would be expected, when the diet was available in quantities desired, the addition of 6 or 9 µg of thiamine to the diet stimulated increases in food intakes; and weight gains in corresponding groups were larger than those observed in the animals on controlled food intakes.

The absence of thiamine from the diet, with its resultant low food intake brought about marked losses in body weight (negative gain per calorie ingested) whether or not vitamin A was present or the animals were on controlled or ad libitum feeding. When both thiamine and vitamin A were present in the diet and the animals consuming their food ad libitum (table 2), 6 and 9 µg of thiamine produced identical gains in weight per calorie ingested. The slightly higher gain per calorie ingested (+ 0.04 gm) at the 3-unit level of vitamin A as compared to the response at either ½, or 1½ I.U. of vitamin A (+ 0.04 gm) may indicate a growth stimulating action of vitamin A. The gain per calorie ingested was lower on the limited calorie intake of the controlled feeding group (table 1) than for corresponding groups eating ad libitum. However, here again is some evidence of the growth promoting function of vitamin A in that 3 and 1½ I.U. of vitamin A produced a positive gain per calorie ingested whereas ½ I.U. of vitamin A produced a negative gain per calorie ingested on practically identical caloric intakes.

The daily addition of ½, 1½, or 3 I.U. vitamin A to the diet, in the absence of thiamine did not produce significant changes in food intakes or weight losses in the animal eating ad libitum as indicated by "t" tests and as shown in table 2. In groups of animals consuming controlled diets, however, the food intakes were somewhat lower and the weight losses greater (in the triads receiving ½ and 3 I.U. of vitamin A daily) than when neither vitamin A nor thiamine were present. In each case, 6 animals made up the group and it is likely that these numbers are too small for more than indications, but it is possible that an imbalance of vitamins with limited total caloric intake led to a poorer response to the food available.

TABLE 2

Ad libitum feeding group: Average weights, food consumption, and autopsy findings on animals on a 6-week experimental period.

VITAMIN LEVELS		NUMBER OF RATS	WEEKLY AVERAGE			GAIN PER CALORIE INGESTED	LENGTH OF RAT AT END OF EXPERIMENT [1]	"ABSCESSES" EVIDENT AT AUTOPSY [2]
Thiamine	Vitamin A		Food intake [1]	Calorie intake	Weight change [1]			
μg	I.U.		gm		gm	gm	in.	no.
0	0	6	11.5 ± 1.7	47.2	− 12.6 ± 1.2	− 0.27	11.8 ± 0.0	3.2 ± 0.8
6	0	6	26.9 ± 1.9	110.3	− 1.8 ± 2.0	− 0.16	12.9 ± 0.9	5.2 ± 0.7
9	0	6	23.9 ± 1.9	98.0	− 4.5 ± 1.9	− 0.46	12.4 ± 0.9	5.7 ± 0.7
18	0	5	29.9 ± 3.4	122.6	+ 0.1 ± 2.5	+ 0.01	13.9 ± 0.0	3.8 ± 1.0
0	½	4	10.9 ± 0.9	44.7	− 12.9 ± 1.5	− 0.29	12.0 ± 0.6	3.3 ± 0.9
6	½	4	33.8 ± 1.8	138.6	+ 5.0 ± 1.2	+ 0.04	12.5 ± 0.4	1.5 ± 0.8
9	½	4	33.6 ± 1.8	137.8	+ 5.1 ± 1.9	+ 0.04	12.3 ± 0.5	1.3 ± 0.3
18	½	1	23.2 ± 2.9	95.1	+ 2.3 ± 2.0	+ 0.02	11.5 ± 0.0	1.0 ± 0.0
0	1½	6	11.0 ± 1.2	45.1	− 11.6 ± 1.3	− 0.26	12.5 ± 0.0	1.3 ± 0.6
6	1½	6	44.3 ± 0.8	181.6	+ 8.1 ± 0.7	+ 0.04	13.6 ± 0.1	1.7 ± 0.4
9	1½	6	44.0 ± 1.4	180.4	+ 7.9 ± 1.3	+ 0.04	13.7 ± 0.2	1.5 ± 0.5
18	1½	3	39.7 ± 0.9	162.8	+ 6.2 ± 1.6	+ 0.04	13.3 ± 0.6	3.7 ± 0.7
0	3	3	11.4 ± 3.0	46.7	− 10.9 ± 1.7	− 0.23	12.9 ± 0.0	2.7 ± 0.9
6	3	3	41.3 ± 2.1	169.3	+ 7.9 ± 1.5	+ 0.05	13.4 ± 0.2	0.3 ± 0.3
9	3	3	47.3 ± 2.6	193.9	+ 9.5 ± 2.0	+ 0.05	13.8 ± 0.0	0.0 ± 0.0

[1] Arithmetic means ± standard error.

[2] Areas that most frequently showed "abscesses" were middle ear, base of tongue, lymph glands in neck, salivary glands, and genito-urinary tract.

When the relation of the response of the animals to the addition of vitamin A with known quantities of thiamine was tested statistically, vitamin A seemed to function as an essential factor in weight increases. In groups of animals fed ad libitum significant increases in food intake and decreases in weight loss or actual gains occurred with the addition of ½, 1½, and 3 I.U. of vitamin A with all corresponding levels of thiamine. In groups of animals with controlled food consumption, however, there were usually not significant increases in food intakes with the addition of vitamin A, but there were consistent and significant weight increases. Patterson, Henry and Crandall ('42) reported that rats which were pair-fed and received vitamin A were significantly heavier than control animals deprived only of that vitamin. The difference in weight was due to a greater retention of water, fat and protein in the animals supplied with the vitamin, and apparently was not due to alterations in absorption of food. In the present controlled-feeding groups, the difference between the average weekly weight changes in animals receiving 9 μg of thiamine and no vitamin A (— 2.7 gm) and those receiving 9 μg of thiamine and 3 I.U. vitamin A (+ 0.8 gm) was 3.5 gm. On the other hand, the difference between the average weekly weight gain of animals receiving 3 I.U. of vitamin A and no thiamine (— 11.6 gm) and those receiving 3 I.U. of vitamin A and 9 μg of thiamine (again + 0.8 gm) was 12.38 gm. A consideration of these differences in weight change, as ratios, seemed to indicate that both vitamins are essential for weight increases but that thiamine seemed to produce between 3 and 4 times the increase that vitamin A produced under the conditions of this experiment and in a 6-week period. It would be essential to know the composition of these weight changes before their relation to growth could be stated.

No significant effect on the length of the animal was observed with any of the different combinations of vitamin intake.

For all groups of animals, "abscesses" were highest in number when no vitamin A was present in the diet, regardless of

the presence of thiamine. In the controlled diet groups (table 1), "abscesses" averaged 3.2 per rat with no vitamin A or thiamine. The number of "abscesses" among animals eating ad libitum (table 2) averaged 3.2 per rat with no vitamin A or thiamine. When no vitamin A was fed, there were significant increases in the number of "abscesses" with the addition of thiamine in most groups of animals. In most instances, however, the addition of thiamine plus vitamin A was more effective in reducing the number of "abscesses" than was vitamin A alone. Since the presence or absence of "abscesses" is probably the most critical measure of vitamin A utilization applied in this study, the findings on the incidence of "abscesses" seems to indicate a synergistic effect of the 2 vitamins under discussion.

Thiamine independently and predominantly stimulated weight changes. Vitamin A, however, was also functioning as a weight-increase factor, at least in the presence of thiamine, but at what might be termed a "slower rate." The reasons for the slightly lower food intakes and greater weight losses among animals fed iso-caloric diets with vitamin A and no thiamine are not clear from the criteria used in this study, but are probably not significant. That there were synergistic effects of thiamine on the utilization of vitamin A in vitamin A depleted rats were not strikingly demonstrated by these data, although they seem to be indicated in the findings on the incidence of "abscesses."

SUMMARY

The effect of thiamine on the utilization of vitamin A was studied by means of food intake, weight change, length of rat, and number of foci of keratinized epithelial tissues called "abscesses" in animals depleted of vitamin A.

The daily addition of 6, or 9 μg of thiamine to the diet, whether or not vitamin A was present, significantly increased food intakes and favorably affected weight changes in all groups of animals. On the other hand, the daily addition of ½, 1½, or 3 I.U. of vitamin A, in the absence of thiamine, did

not produce significant changes in food intakes or weight losses in animals eating ad libitum of a basal vitamin A and thiamine free diet. In groups of animals on iso-caloric intakes, however, food intakes were somewhat lower and weight losses greater when ½ or 3 I.U. of vitamin A were given, than when neither vitamin A nor thiamine were present in the food. Vitamin A functioned as an essential factor in weight increases in the presence of thiamine.

No significant differences in the length of the rats were observed among the groups, regardless of vitamin intake in the 6-week period.

For all groups of animals, "abscesses" were highest in number when no vitamin A was present in the diet. When no vitamin A and some thiamine were fed, there were significant increases in the number of "abscesses" in several groups of animals. In most instances, however, the addition of thiamine plus vitamin A was more effective in reducing the number of "abscesses" than was vitamin A alone.

LITERATURE CITED

DYE, M., I. BATEMAN AND T. PORTER 1945 The effect of the level of protein in the diet on the utilization of vitamin A. J. Nutrition, *29:* 341.

MITALO, M. 1942 Nuovi studi sulle correlazioni intervitaminiche (New studies on the correlation of vitamins) (2) Axeroftolo e B-carotene nel fegate di ratti avitaminosi A B_1 Boll. Soc. Ital. Biol. sper., *17:* 311.

MUELDER, K. D., AND E. KELLY 1941 The effect of the quality of basal food intake upon the utilization of vitamin A. J. Nutrition, *21:* 13.

———— 1942 The effect of the level of fat in the diet upon utilization of vitamin A. J. Nutrition, *23:* 335.

PATTERSON, J. M., E. W. MCHENRY AND W. A. CRANDALL 1942 The physiological properties of vitamin A. 1. A specific effect upon body weight and body composition in the albino rat. Biochem. J., *36:* 792.

RANDOIN, L., AND R. NETTER 1936 Différence profonde entre le degré de necessité des vitamines A et B, à une époque précise de la croissance du jeune rat, et dans le cas d'un régime très riche en glucides. Compt. rend., *202:* 1105.

SCHEUNERT, A., AND A. RAU 1937 Über den Einfluss verschiedener Vitamin A-Mengen auf den Vitamin B_1-Bedarf. Mitteilung zum Synergismus der Vitamine. Z. Physiol. Chem., *246:* 267.

SCHEUNERT, A., AND L. WOLFANGER 1938 Über den Einfluss verschiedener Vitamin B_1-Mengen auf den Vitamin A-Bedarf. (Wechselwirkung der Vitamine. 2.) Z. Physiol. Chem., *252:* 95.

THE VALUE OF MEAT AND PEAS, ALONE OR IN COMBINATION, AS A SOURCE OF PROTEIN FOR GROWTH [1]

W. P. LEHRER, JR., ELLA WOODS AND W. M. BEESON [2]
Departments of Animal Husbandry and Home Economics, Idaho Agricultural Experiment Station, Moscow

(Received for publication October 25, 1946)

The proteins of meat are considered of high nutritive quality. Hoagland and Snider ('26, '27) reported them to be adequate for growth in rats when they supplied protein at a 10% level and to be very efficient supplements for the cereals and for white flour. Clayton ('30), also working with rats, obtained growth, maintenance and reproduction with beef protein at a 20% level. McCollum et al. ('21) found meats valuable supplements for cereal seeds but not so satisfactory as supplements for legume seeds.

Woods et al. ('43) reported that the slow growth in rats on the protein of raw Alaska peas could be overcome by the addition of methionine. Bolin and associates ('46) found that for chick growth, various animal and plant protein supplements to pea meal rations were greatly improved by the addition of methionine. Recent investigations at this station by Beeson and Hickman ('45, '45a) and Lehrer et al. ('46, '46a) have shown that peas, when supplemented with a small quantity of meat meal or good pasture, are an excellent source of protein when fattening swine.

[1] Published with the approval of the Director of the Idaho Agricultural Experiment Station as Research Paper no. 265.

[2] Present address: Department of Animal Husbandry, Purdue University, Lafayette, Indiana.

In low-cost diets or when meats are difficult to obtain, a very common dietary recommendation is an increased use of legumes to meet the protein requirement. The study reported here was designed to compare, at a critical level, the proteins of pork, lamb, beef and peas and to determine the effect upon growth of replacing a part of the meat of the diet with a serving of peas.

EXPERIMENTAL PROCEDURE

Weanling rats housed in individual cages and weighed weekly for 8 weeks were used as test animals. Food consumption records were made at each weighing.

Alaska field peas, grade no. 1, were soaked, cooked and dried without discarding any of the liquid and then finely ground.

The meats were prepared and roasted as recommended by the National Livestock and Meat Board ('45). The internal temperatures of the various cuts were as follows: for fresh ham, 185°F.; leg of lamb, 180°F.; and beef round, 170°F. All bone and all visible fat were removed from the meats before they were ground. They were dehydrated at a temperature of 144°F. in the same manner as the peas and were stored under refrigeration. A diet in which dried whole egg furnished the protein was used in these experiments as a standard of comparison. Animals on the egg diet exhibited good growth with efficient utilization of protein.

The dried cooked peas and the dried meats were analyzed for nitrogen and fat. The diets were made up to contain 10% protein ($N \times 6.25$) and were adjusted to the same fat level by the use of refined cottonseed oil. The composition of the diets is given in table 1.

For compounding the diets in the study of peas as an extender of meats, 50 gm of peas and 4 oz. of fresh meat were taken as average servings of these foods in human dietaries. In those diets marked "a" the protein was derived from a mixture of peas and meat in the relationship of 1 serving of peas (50 gm) to ¼ serving of fresh meat (1 oz.). In those

TABLE 1

Composition of diets.[1]
(Source of protein calculated to 10% protein level.)

LOT NO.	PROTEIN SOURCE		SUGAR	SALT MIXTURE	COTTON-SEED OIL	COD LIVER OIL	AGAR-AGAR
		gm/kg	*gm/kg*	*gm/kg*	*gm/kg*	*gm/kg*	*gm/kg*
1	Egg	227	639.5	40	30	20	20
2	Pork	154	738	40	4.5	20	20
3	Lamb	135	729.5	40	32	20	20
4	Beef	142	723	40	31.5	20	20
5	Peas	433	444.5	40	39	20	..
6	Peas and dl methionine	433 3	441.5	40	39	20	..
7	Peas and Pork (a)	320 40	526.5	40	30	20	..
8	Peas and Pork (b)	254 63	575	40	24.5	20	..
9	Peas and Lamb (a)	310 39	530.5	40	37	20	..
10	Peas and Lamb (b)	241 60	580	40	35.5	20	..
11	Peas and Beef (a)	314 39	526.5	40	37	20	..
12	Peas and Beef (b)	246 61	574	40	35.5	20	..

[1] Each diet contained 21 gm/kg of vitamin-sugar mixture in addition to 2.5 gm/kg of choline chloride.

(a) Mixed in proportion of ¼ serving (1 oz.) of *fresh* meat to 1 serving (50 gm) of peas.

(b) Mixed in proportion of ½ serving (2 oz.) of *fresh* meat to 1 serving (50 gm) of peas.

marked "b" the relationship was that of 1 serving of peas to ½ serving (2 oz.) of fresh meat. The protein of these mixtures was fed at the 10% level as in the other diets.

Pure vitamins of the B-complex were mixed with sugar, and 21 gm of the mixture included in each kilo, providing the following supplements per 100 gm of diet: thiamine, 0.49 mg; riboflavin, 0.49 mg; pyridoxine, 0.61 mg; nicotinic acid, 0.61 mg; calcium pantothenate, 4.9 mg; para-amino benzoic acid, 29.4 mg; and inositol, 98.0 mg. The Osborne and Mendel ('19) salt mixture was used in all diets.

Statistical analyses of the data were made according to the methods of Snedecor ('40).

RESULTS AND DISCUSSION

The results of this series of experiments are summarized in tables 2 and 3.

On the basis of daily gains in weight the pork, lamb, and beef compared favorably with egg, but the peas were significantly inferior (table 3). From the standpoint of efficiency as measured by gain per gm of protein consumed, pork, lamb and beef were not significantly different from egg, but the peas were markedly less efficient.

When cooked peas were supplemented with 0.3% dl-methionine the average daily gain was tripled and the protein efficiency was more than doubled, confirming earlier published findings of Woods and co-workers ('43) that peas lack methionine for normal growth. Although the peas and methionine diet was not as efficient as the whole egg diet, the daily gains were not significantly different.

The rats on the pea diet, in addition to being smaller, had short, soft hair and their average survival period was only 33 days. A postmortem [3] examination revealed a characteristically abnormal liver. The liver condition was typical of acute hepatitis and characterized by 2 progressive degrees of involvement — cloudy swelling and fatty degeneration.

[3] Credit is due Dr. G. C. Holm, Station Veterinarian, for valuable assistance in postmortem studies.

The stage of liver damage was dependent upon the length of time the rat showed retarded growth. No cases of cirrhosis of the liver were observed, which can be attributed to the acute nature of the involvement. Occasionally hemorrhage in the liver was encountered. These conditions did not occur in rats receiving the pea diet supplemented with adequate methionine.

In diets in which the peas and meats were combined, at both levels of meat, the average daily gain and the efficiency of the

TABLE 2

Average growth response.

LOT NO.	DIET	NO. OF RATS	AV. DAILY GAIN	AV GAIN PER GM OF PROTEIN	AV. FOOD REQUIRED PER GM GAIN	AV. FOOD CONSUMED DAILY	AV. LIFE OF RATS [1]
			gm	*gm*	*gm*	*gm*	*days*
1	Egg	8	2.17 ± .11	2.50 ± .08	3.99 ± .20	8.65 ± .36	56
2	Pork	9	2.00 ± .11	2.16 ± .10	4.63 ± .24	9.28 ± .26	56
3	Lamb	9	2.34 ± .12	2.32 ± .11	4.31 ± .26	10.09 ± .31	56
4	Beef	9	2.35 ± .09	2.19 ± .05	4.56 ± .11	10.75 ± .33	56
5	Peas	19	0.60 ± .04	0.95 ± .06	10.54 ± .68	6.36 ± .45	33
6	Peas and 0.3% dl-methionine	19	1.88 ± .09	2.04 ± .07	4.89 ± .13	9.20 ± .39	56
7	Peas and Pork (a)	9	1.38 ± .17	1.94 ± .24	5.16 ± .66	7.15 ± .43	21
8	Peas and Pork (b)	9	1.62 ± .10	1.70 ± .10	5.88 ± .30	9.54 ± .59	42
9	Peas and Lamb (a)	9	1.66 ± .13	2.41 ± .07	4.15 ± .13	6.89 ± .43	21
10	Peas and Lamb (b)	9	1.80 ± .08	1.89 ± .14	5.30 ± .36	9.51 ± .56	41
11	Peas and Beef (a)	9	1.38 ± .19	1.96 ± .17	5.10 ± .70	7.03 ± .54	25
12	Peas and Beef (b)	9	1.85 ± .03	1.95 ± .15	5.42 ± .33	10.00 ± .55	48

[1] Experimental period 56 days.

TABLE 3

Statistical comparisons of growth on various diets.

LOT NO.	DIETS COMPARED	AVERAGE DAILY GAIN (GM)			AVERAGE GAIN PER GRAM PROTEIN (GM)		
		Mean difference	Standard error of difference	t [1]	Mean difference	Standard error of difference	t [1]
1–2	Egg vs. Pork	0.17	.16	1.06	0.34	.13	2.66
1–3	Egg vs. Lamb	0.17	.16	1.06	0.18	.14	1.28
1–4	Egg vs. Beef	0.18	.14	1.28	0.31	.09	3.44
1–5	Egg vs. Peas	1.57	.12	13.08	1.55	.10	15.50
2–3	Pork vs. Lamb	0.34	.16	2.13	0.16	.15	1.07
2–4	Pork vs. Beef	0.35	.14	2.50	0.03	.11	0.03
7–9	Peas + Pork (a) vs. Peas + Lamb (a)	0.28	.22	1.27	0.47	.25	1.88
7–11	Peas + Pork (a) vs. Peas + Beef (a)	0.00	.25	0.00	0.02	.29	0.06
8–10	Peas + Pork (b) vs. Peas + Lamb (b)	0.18	.13	1.38	0.19	.17	1.12
8–12	Peas + Pork (b) vs. Peas + Beef (b)	0.23	.10	2.30	0.25	.18	1.39
5–6	Peas vs. Peas + 0.3% dl-methionine	1.28	.10	12.80	1.09	.09	12.11
1–6	Egg vs. Peas + 0.3% dl-methionine	0.29	.14	2.07	0.46	.11	4.18
6–7	Peas + 0.3% dl-methionine vs. Peas + Pork (a)	0.50	.19	2.63	0.10	.25	0.40
6–8	Peas + 0.3% dl-methionine vs. Peas + Pork (b)	0.26	.13	2.00	0.34	.12	2.83
6–9	Peas + 0.3% dl-methionine vs. Peas + Lamb (a)	0.22	.16	1.38	0.37	.10	3.70
6–10	Peas + 0.3% dl-methionine vs. Peas + Lamb (b)	0.08	.12	0.67	0.15	.16	0.94
6–11	Peas + 0.3% dl-methionine vs. Peas + Beef (a)	0.50	.21	2.38	0.08	.18	0.44
6–12	Peas + 0.3% dl-methionine vs. Peas + Beef (b)	0 03	.09	0.33	0.09	.17	0.53

[1] A t value of 3.499 establishes the 1% point for the comparisons based on the smallest number of degrees of freedom; therefore a value of 3.499 or larger is highly significant.

proteins were significantly increased over diets in which peas alone supplied the protein. The peas and meat combination diets gave average daily gains that were not significantly different from those obtained with the peas and methionine diet but on none of the pea and meat combination diets did all the animals live the entire test period. On the lower level of meat the average survival time was less than ½ the test period and on the higher level 6 to 7 weeks. Rats on these diets made good growth but died suddenly without previous indications of disorder. On autopsy they revealed a liver condition of varying severity similar to the one found in the rats on peas.

Since this liver condition was prevented by adequate methionine supplements to the pea diet it appears that the meats in the amounts here used did not furnish sufficient methionine to provide for the increased growth and for maintenance of normal liver tissue.

If the methionine content of voluntary muscle protein is estimated at 3%, which is approximately the value reported by Beach et al. ('43) from chemical analyses of beef, pork and lamb muscle, the amounts of methionine added to each kilo of diet by the meat supplements actually used in these experiments were about 0.8 gm by the smaller and 1.3 gm by the larger supplements. These values are considerably below the 3 gm per kilo added in the 0.3% dl-methionine supplemented pea diet. Mitchell and Block ('46) in their comparison of analytical data for different food proteins with those of dried whole egg note that for beef muscle the indicated limiting amino acid is the methionine plus cystine combination. These figures suggest that the peas and meat combination diets at both levels of supplementary meat were limited by their content of methionine but do not explain the acceleration of growth at the expense of liver tissues.

SUMMARY

Cooked pork, lamb, beef, and peas, and some combinations of peas and the meats, have been compared with whole egg as sources of protein for growth in young rats.

Measured by growth rate and by efficiency the various meats, when fed at a 10% protein level, were as good sources of protein for growth as were eggs.

Cooked peas alone were a poor source of protein for growth whether measured by average daily gain or by gain per gram of protein. When peas were supplemented with 0.3% dl-methionine, growth was increased until it was not significantly different from that obtained with whole egg protein although it was not as efficient.

At the levels of meat fed in combination with peas in this investigation growth was improved over that from peas alone but even the meat and pea diets were inadequate as evidenced by a characteristic liver involvement and early death of many of the test animals.

LITERATURE CITED

BEACH, E. F., B. MUNKS AND A. ROBINSON 1943 The amino acid composition of animal tissue protein. J. Biol. Chem., *148:* 431.

BEESON, W. M., AND C. W. HICKMAN 1945 Cull peas as a protein supplement for fattening hogs. Idaho Agr. Exp. Sta. Cir. 105, 4 pp.

——— 1945a Nutritive value of cull peas for fattening hogs. Idaho Agr. Exp. Sta. Cir. 106, 4 pp.

BOLIN, D. W., C. F. PETERSON, C. E. LAMPMAN AND O. E. STAMBERG 1946 Chick growth response resulting from methionine additions to various protein supplements with pea proteins. Poultry Sci., *XXV:* 157.

CLAYTON, M. M. 1930 The comparative value of different food proteins for reproduction and lactation in the rat. I. Beef muscle, liver and kidney. J. Nutrition, *2:* 491.

HOAGLAND, R., AND G. G. SNIDER 1926 Nutritive value of the protein in voluntary muscle, heart, liver, and kidney, from cattle, sheep and hogs. J. Agr. Res., *32:* 1025.

——— 1927 The value of beef protein as a supplement to the proteins in certain vegetable products. J. Agr. Res., *34:* 297.

LEHRER, W. P., JR., AND C. W. HODGSON 1946 Cull peas for fattening hogs in dry lot. Idaho Agr. Exp. Sta. Cir. 107, 4 pp.

LEHRER, W. P., JR., W. M. BEESON AND A. WILSON 1946a Cull peas as a protein supplement for fattening hogs on pasture. Idaho Agr. Exp. Sta. Cir. 108, 4 pp.

MCCOLLUM, E. V., N. SIMONDS AND H. T. PARSONS 1921 Supplementary protein values in foods. II. Supplementary dietary relations between animal tissues and cereal and legume seeds. J. Biol. Chem., *47:* 139.

MITCHELL, H. H., AND R. S. BLOCK 1946 Some relationships between the amino acid content of proteins and their nutritive values for the rat. J. Biol. Chem., *163:* 599.

NATIONAL LIVESTOCK AND MEAT BOARD 1945 Meat Manual, Chicago, Ill.
OSBORNE, T. B., AND L. B. MENDEL 1919 The nutritive value of the wheat kernel and its milling products. J. Biol. Chem., *37:* 557.
SNEDECOR, G. W. 1940 Statistical Methods. Iowa State College Press, Ames, Iowa, p. 58.
WOODS, E., W. M. BEESON AND D. W. BOLIN 1943 Field peas as a source of protein for growth. J. Nutrition, *26:* 327.

TISSUE RESERVES OF ASCORBIC ACID IN NORMAL ADULTS ON THREE LEVELS OF INTAKE [1]

J. ESTELLE HAINES, ANN MOORE KLOSTERMAN, HAZEL M. HAUCK, MARGARET A. DELANEY AND ALICE B. KLINE,

WITH THE COOPERATION OF ELEANOR B. SISLER AND MARGARET NEWELL

New York State College of Home Economics, and School of Nutrition, Cornell University, Ithaca, New York

ONE FIGURE

(Received for publication November 27, 1946)

Nutrition workers are not yet agreed on a desirable nutritive state with respect to vitamin C, nor on the intake of ascorbic acid which should be recommended to maintain such a state. The recommended allowance of the National Research Council, 70 mg for women and 75 mg for men, is insufficient to maintain tissue saturation in adults but provides a wide margin of safety over the amount required for prevention of scurvy. The League of Nations standard allowance of 30 mg daily has been commonly accepted in Europe, and is recognized in the United States as the minimum requirement to be stated in food labeling.

The present study is concerned with a comparison of tissue stores of ascorbic acid associated with 3 levels of intake which had been maintained for a period of 6 weeks. The intakes used were 70 mg, 50 or 53 mg, and 33 mg, respectively. The

[1] Some of the data in this paper are taken from theses presented to the faculty of the Graduate School of Cornell University in fulfillment of the requirement for the M.S. degree, by Ann C. Moore, June 1944, Margaret A. Delaney, October 1945, and J. Estelle Haines, February 1946. Further details will be found in the theses.

highest level of intake studied approximated the National Research Council allowance, the lowest approximated the League of Nations standard, whereas in the other 2 experiments, 1 with natural food and the other with a synthetic diet, the intakes were intermediate.

The state of the subjects' tissue reserves of ascorbic acid at the end of the 6-week periods was judged by the fasting plasma ascorbic acid values and whether or not these values had reached a plateau, as well as by the response to standard test doses, and the number of doses given daily which were required to restore tissue saturation.

The experimental period selected, 6 weeks, is one in which the fasting plasma ascorbic acid value reached zero for a subject whose diet was devoid of ascorbic acid (Crandon, Lund and Dill, '40), hence it was believed to be long enough to permit the demonstration of differences in body stores of ascorbic acid on these levels of intake, should such differences exist.

EXPERIMENTAL

Subjects

Twelve normal adults, 6 men and 6 women, ranging in age from just under 20 to 46 years, served as subjects. Four of these served for 2 experimental periods each. Data concerning the sex, age, height and weight of the subjects are presented in table 1. Before each experiment, the subjects were examined by a physician at the Student Medical Clinic of Cornell University and were found to be in good physical condition.[2] Six of the subjects had mild infections of the upper respiratory tract,[3] without fever, in the course of the experiments which were conducted during the winter. Subject J. M. had a cold with slight fever (99.6°F.) for 3 days during the sixth week of the experiment.

[2] Subjects E.K. and H.G. had poliomyelitis in childhood; subject J.M. had asthma in 1931, with little difficulty since.

[3] Although the subjects had been instructed not to take any medication during the experimental period, 1 subject took 110 grains of aspirin in a 32-hour period in the fifth week of the experiment, without apparent effect on the urinary excretion or fasting plasma values of ascorbic acid.

TABLE 1

Sex, age, height and weight of 12 subjects[1] on controlled intakes of ascorbic acid for 6-week periods.

SUBJECT	AGE	HEIGHT		WEIGHT[2]		SUBJECT	AGE	HEIGHT		WEIGHT[2]	
	yr.	*inches*	*cm*	*lb.*	*kg*		*yr.*	*inches*	*cm*	*lb.*	*kg*
	Intake: 33 mg/day						*Intake: 53 mg/day*				
EH ♀	22	66½	169	134	61	AM ♀	23	67½	172	136	62
SP ♂	19	67	170	140	64	DL ♀	30	66	168	143	65
HG ♂	26	72½	184	127	58	EK ♂	24	69	175	154	70
LG ♀	29	67	170	153	69	HG ♂	26	72½	184	133	60
	Intake: 50 mg/day						*Intake: 70 mg/day*				
JES ♀	21	63	160	128	58	AM ♀	22	67½	172	134	61
DL ♀	31	66	168	133	60	PH[3] ♂	31	68½	174	141	64
MH ♀	46	67	170	138	63	EK ♂	23	69	175	163	74
						JM ♂	24	68	173	163	74
						JL ♂	23	67	170	152	69

[1] Subjects HG, DL, AM and EK served for 2 experimental periods.

[2] Average of daily weights during 6-week period. Nude weight obtained by subtracting the weight of the usual amount of clothing from the daily weight.

[3] Subject PH is Chinese.

Diet and ascorbic acid intake

The basal diet for 3 of the 4 experimental periods was similar to that reported by Lewis et al. ('43). This diet provided from 10 to 13 mg of ascorbic acid, and was supplemented by doses of 20, 40 and 60 mg of synthetic ascorbic acid,[4] taken after breakfast. These doses, together with the ascorbic acid content of the basal diet, brought the total ascorbic acid intake to 33, 53 and 70 mg, respectively, in the 3 experiments. In the study on the 53-mg intake, the diet was modified so as to keep the thiamine intake constant for each subject (Gifft and Hauck, '46). In the fourth experiment, which was designed primarily to study thiamine metabolism (Hathaway and Strom, '46) a synthetic diet was used, including 50 mg of ascorbic acid.

[4] Acknowledgment is made to Hoffmann-LaRoche, Inc., for a generous supply of synthetic ascorbic acid.

Plan of the experiment

Preliminary saturation period. In order to assure full tissue stores for all subjects at the beginning of the experiments, they were given either the juice of 8 oranges, or 400 mg ascorbic acid daily for 3 days in addition to their usual diets. On the fourth day, subjects were given either the basal diet, or other food selected for its low ascorbic acid content, plus a test dose of 400 mg of ascorbic acid, 200 mg with breakfast and 200 mg with luncheon. Urinary excretions of ascorbic acid on this day were from 250 to 387 mg, well in excess of 50% of the test dose. Fasting plasma ascorbic acid values were 1.2 mg % or more for all subjects at this time.

Experimental period. The preliminary saturation period was followed by a period of 6 weeks on the level of intake under investigation, i.e., 33, 50, 53 and 70 mg per day, respectively, in the 4 experiments.

Resaturation period. Following the period of 6 weeks on a constant level of intake, subjects were given daily 400 mg test doses of ascorbic acid, 200 mg with breakfast and 200 mg with luncheon, until the 24-hour urinary excretion of ascorbic acid exceeded 50% of the test dose. The subjects were then considered to be saturated with respect to ascorbic acid.

Ascorbic acid determinations made. Daily determinations were made of the ascorbic acid content of the fasting plasma and 24-hour urine samples. Each 24-hour period ended with the collection of the specimen voided ½ hour before the fasting blood sample was taken. The fasting plasma level was considered to be associated with the previous day's intake; therefore, in making calculations, the fasting plasma values were paired with the previous day's urinary excretion values.

In order to estimate the subjects' renal thresholds for ascorbic acid, determinations were made of the hourly excretion of ascorbic acid, both at fasting and following ingestion of ascorbic acid, together with the corresponding plasma ascorbic acid values. Some of these data are presented here, since they provide additional evidence concerning the state of the

subjects' tissue stores at the beginning and end of the experiment. The estimation of the renal threshold is reported in a separate publication (Klosterman et al., '47).

Analytical methods

Each urine specimen, or an aliquot portion thereof, was acidified immediately with 10% by volume of 2 N sulfuric acid containing 2% of metaphosphoric acid. The acidified 24-hour specimens, or appropriate aliquot portions thereof, were refrigerated and pooled for analysis. The ascorbic acid content of the urine was determined by titration with a buffered solution of the indicator, sodium 2, 6-dichlorobenzenone-indophenol,[5] standardized twice weekly against a known solution of ascorbic acid.

Reduced ascorbic acid in plasma was determined by the micromethod of Farmer and Abt ('36).

The ascorbic acid content of foods in the basal diet was determined 3 or more times during each experimental period, using the indophenol titration method of Bessey ('38a) modified by the use of the Waring Blendor (Davis, '39).

RESULTS AND DISCUSSION

The results of these experiments are summarized in tables 2 and 3.

Evidence on the tissue reserves, obtained from the 24-hour excretion of ascorbic acid, was corroborated by the hourly determinations of urinary excretion and plasma levels of ascorbic acid, made in connection with the estimation of the subjects' renal thresholds. Typical curves obtained from such studies are presented in figure 1, A and B. Curves marked 1 are representative of values obtained on the first day of the 6-week period. The fasting plasma values for all subjects were near their renal thresholds for ascorbic acid, and were maintained above the threshold value for several hours by the small daily supplement. This behavior is typical of subjects

[5] Eastman Kodak Company.

TABLE 2

Fasting plasma ascorbic acid values for adult subjects on various levels of intake during a 6-wee and in response to 400-mg test doses at the end of the period.

INTAKE AND SUBJECT	MEAN FASTING PLASMA ASCORBIC ACID VALUES IN MG %						RESPONSE TO 400 MG TEST DOSE FASTING PLASMA ASCORBIC ACI VALUES, MG %				
	Week[2]						Day				
	1	2	3	4	5	6	1	2	3	4	5
70 mg											
A.M.♀	1.07	1.00	0.89	0.86	0.89	0.86	1.5	1.3			
P.H.♂	0.76	0.90	0.66	0.67	0.70	0.67	1.1	1.1			
E.K.♂	0.89	0.76	0.64	0.61	0.57	0.50	0.5	1.0	1.3	1.2	
J.M.♂	1.06	0.94	0.70	0.63	0.63	0.63[3](6)	0.9	1.2	1.1		
J.L.♂	0.83	0.76	0.61	0.67	0.66	0.66	1.1	1.2			
53 mg											
A.M.♀	1.22	0.83	0.77	0.74	0.63	0.63	1.0	1.3	1.5		
E.K.♂	1.03	0.67	0.57	0.48	0.44	0.42	0.6	0.9	1.2	1.2	1.4
H.G.♂	1.04	0.71	0.61	0.53	0.48	0.46	1.1	1.1	1.3	1.4	
D.L.♀	1.25	0.85	0.78	0.69	0.61	0.53	1.1	1.4	1.6		
50 mg[4]											
D.L.♀	1.16(5)	0.98(5)	0.94(5)	0.81	0.84	0.59	1.0	1.3	1.3		
J.E.S.♀	1.15(6)	1.03(6)	1.02(4)	0.83(6)	0.77	0.59	1.1	1.4	1.4		
M.H.♀	0.93(6)	0.73(6)	0.73(3)	0.63	0.60	0.50	0.8	1.0	0.9	1.1	1.0
33 mg											
E.H.♀	1.00	0.69	0.54	0.40	0.37	0.34	0.6	0.9	1.5	1.3	
S.P.♂	0.96	0.60	0.50	0.41	0.34	0.36	0.7	1.2	1.3	1.5	
H.G.♂	0.93	0.63	0.51	0.41	0.37	0.28(6)	0.6	0.7	1.2	1.4	1.5
L.G.♀	0.93	0.61	0.49	0.41	0.34	0.31	0.6	0.8	1.4	[5]	1.6

[1] Renal threshold. Data from Klosterman et al. ('47).

[2] Weekly means were calculated from 7 daily values except where a figure in parentheses is giver dicate that the mean was derived from fewer values.

[3] This subject had a cold with fever for 3 days during the sixth week of the experiment.

[4] Synthetic diet used. In all other studies the basal diet was composed of foods low in ascorbic acid c

[5] This subject had a mild illness without fever on the fourth day of the resaturation period. Si fasting plasma determination was made on this day, the experiment was continued 1 day beyond th when more than 50% of the test dose was excreted in 24 hours.

[6] Fasting plasma ascorbic acid values for this subject were at or above her renal threshold for th 2 days.

whose tissue reserves of ascorbic acid are full. Similar plasma ascorbic acid curves for A.M. and J.L. on the second morning of the resaturation period indicated that their tissue stores had been refilled. This was borne out by the excretion of over

TABLE 3

Twenty-four hour urinary ascorbic acid values for adult subjects on various levels of intake during a 6-week period, and in response to 400-mg test doses at the end of the period.

INTAKE AND SUBJECT	MEAN 24-HOUR URINARY EXCRETION VALUES FOR ASCORBIC ACID IN MG						RESPONSE TO 400 MG TEST DOSES, 24-HOUR URINARY EXCRETION VALUES FOR ASCORBIC ACID IN MG						
	Week[1]						Day						
	1	2	3	4	5	6	1	2	3	4	5	6	7
70 mg													
A.M.♀	49	21	21	24	24	23	115	266					
P.H.♂	53	20	21	22	26	25	67	253					
E.K.♂	43	33	32	30	30	32	36	67	199	294			
J.M.♂	46	19	20	23	26	28[2]	32	190	334				
J.L.♂	41	21	22	25	23	23	125	236					
53 mg													
A.M.♀	40	19	19	19	19	17	28	155	252				
E.K.♂	30	24	25	25(6)	24	22	24	25	39	152	242		
H.G.♂	42	21	22	20	19	17	18	84	182	302			
D.L.♀	51	23	22	21(6)	22	17	27	62	215				
50 mg[3]													
D.L.♀	34(6)	22	17(6)	17	15	15	25	110	222				
J.E.S.♀	34	23	20(6)	21	19	19	24	85	222				
M.H.♀	34	22	16	17	15	14	15	32	61	117	124	148	183
33 mg													
E.H.♀	37(6)	26	27(6)	27	24	22	25	28	129	263			
S.P.♂	28	20	23	21	19	17	24	29	108	263			
H.G.♂	37	23	22(6)	21	17	15	25	23	30	175	212		
L.G.♀	30	19	21	19	18	16	20	21	55	230	295[4]		

[1] Weekly means were calculated from 7 daily values except where a figure in parentheses is given to indicate that the mean was derived from fewer values.

[2] This subject had a cold with fever for 3 days during the sixth week of the experiment.

[3] Synthetic diet used. In all other studies the basal diet was composed of foods low in ascorbic acid content.

[4] This subject had a mild illness without fever on the fourth day of the resaturation period. Since no fasting plasma determination was made on this day, the experiment was continued 1 day beyond the time when more than 50% of the test dose was excreted in 24 hours.

50% of the test dose by these subjects on this day. Note, however, that the low fasting plasma values for subjects E.H. and H.G. following the period on a 33-mg intake, were raised only slightly by the 200 mg test dose on the first day of the resaturation period. On the third day, the test dose caused the plasma level of ascorbic acid to exceed the renal threshold for a short time, but the tissues were still avidly taking up ascorbic acid, so that little urinary excretion occurred. As judged by the 24-hour urinary excretion in response to the test dose E.H. required 1 more, and H.G. 2 more days to become saturated.

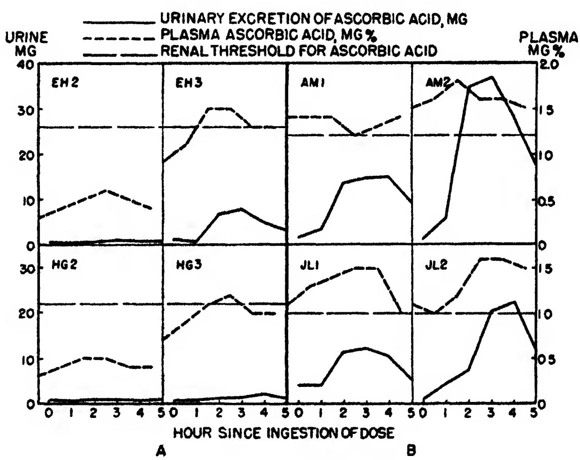


Fig. 1 Plasma ascorbic acid and urinary excretion curves following ingestion of ascorbic acid. In each case the dose of ascorbic acid was taken at the hour marked 0, immediately following the collection of a 1-hour fasting specimen of urine, and ½ hour after the fasting blood sample was taken. Each subject's renal threshold for ascorbic acid is indicated by a line parallel to the base line. Fig. 1 A and B include representative curves for subjects on the 33 mg and 7.0 mg intakes, respectively.

Curves marked 1 represent data obtained on the first day of the experiment, following saturation when A.M. and J.L. received 60 mg doses of ascorbic acid at the hour marked 0.

Curves marked 2 and 3 represent data obtained during the resaturation period, when a 200 mg test dose was given at the hour marked 0. Curves marked E.H. 2, H.G. 2 and E.H. 3, H.G. 3 represent studies made on the first and third mornings of the resaturation period; curves marked A.M. 2 and J.L. 2 represent data obtained on the second morning of the resaturation period.

In comparing the results of experiments on the 3 levels of intake, the consistent behavior of subjects who served for 2 experiments is noteworthy. Thus A.M. required 2 days for resaturation on the 70 mg intake, and 3 days on the 53 mg intake; E.K. required 4 days on the 70 mg intake and 5 days on the 53 mg intake; H.G. required 4 days on the 53 mg intake and 5 days on the 33 mg intake; and D.L. required 3 days for resaturation after periods on 53 and 50 mg intakes. There was no indication that D.L.'s ascorbic acid metabolism on the synthetic diet differed from her metabolism when the basal diet was composed of ordinary foods.

In general, the reducing value of E.K.'s 24-hour specimens of urine was higher than for the other subjects, after the first week of the study. This may have been related to a higher excretion of non-ascorbic acid reducing substances by this subject, since in a series of determinations with the photoelectric colorimeter by the method of Bessey ('38b), the mean urinary ascorbic acid excretion was 14 mg less than when estimated by direct titration, as compared with the following differences for other subjects studied at the same time: P.H., 6; A.M., 7; J.M. and J.L., 9 mg. We have found no constant relationship between the ascorbic acid values as estimated by these 2 methods.

No explanation is apparent for the anomalous behavior of subject M.H. who did not excrete over 50% of the test dose even after 7 days, although her fasting plasma ascorbic acid value reached her renal threshold on the second day, and hovered about this concentration until the experiment was terminated because the subject left town. She was capable of excreting over 50% of the test dose because she did so during the preliminary saturation period.

Although the 70 mg intake of ascorbic acid was not sufficient to maintain any of the 5 subjects in a state of tissue saturation, 4 of the 5 reached a plateau in urinary excretion of ascorbic acid after the first week and in fasting plasma ascorbic acid values after the second week on this intake (tables 2 and 3). Probably these subjects had reached a stable

state in which the tissue reserves were being maintained at a level slightly below saturation. The prompt response of both plasma and urinary excretion values during the resaturation period indicates that except for subject E.K., these adults had suffered only slight depletion of their tissue stores of ascorbic acid in 6 weeks on the 70 mg intake. In a large scale experiment, Linghorne et al. ('46) found that slightly higher intakes, approximately 75 and 80 mg per day, maintained plasma values between 0.7 and 1.0 mg % in young R.C.A.F. personnel for a period of 8 months.

In contrast to the relatively good stores on the intake which approximated the National Research Council allowance of ascorbic acid, marked depletion of the tissue stores occurred in 6 weeks on the 33 mg intake, which approximates the League of Nations standard. This depletion may not have reached its maximum for this intake, for the fasting plasma ascorbic acid and urinary excretion values had not reached a definite plateau in the 6 weeks (tables 2 and 3). Possibly the values would not have fallen much lower had the experiment been continued, for Linghorne et al. ('46) observed only slightly lower plasma values, approximately 0.25 mg % for subjects who were maintained on a 25 mg intake for 8 months.

As would be expected, the results on the intermediate intakes were intermediate between those on the intakes approximating the National Research Council allowance and the League of Nations standard. These experiments provide no evidence concerning the value of tissue reserves of ascorbic acid from the standpoint of health.

SUMMARY

The ascorbic acid metabolism of normal adults was studied on 3 levels of intake for periods of 6 weeks, followed by resaturation periods. Five subjects received the 70 mg intake, which approximated the National Research Council allowance; 6 subjects received either an intake of 50 mg of ascorbic acid as part of a synthetic diet, or 53 mg, with a basal diet of ordinary foods, 1 subject serving for both experiments; 4

subjects received an intake of 33 mg, which approximated the League of Nations standard allowance. Depletion of tissue stores was slight on the 70 mg allowance, moderate on the allowance of 50 to 53 mg, and fairly marked on the 33 mg intake, without evidence that the maximum depletion had been reached at the end of the 6-week period on this intake.

LITERATURE CITED

BESSEY, O. A. 1938a Vitamin C. Methods of assay and dietary sources. J. Am. Med. Assoc., *111:* 1290.

——— 1938b A method for the determination of small quantities of ascorbic acid and dehydroascorbic acid in turbid and colored solutions in the presence of other reducing substances. J. Biol. Chem., *126:* 771.

CRANDON, J. H., C. C. LUND AND D. B. DILL 1940 Experimental human scurvy. New England J. Med., *223:* 353.

DAVIS, W. B. 1939 A substitute for the laboratory food grinder. Ind. Eng. Chem. (News Ed.), *17:* 752.

FARMER, C. J., AND A. F. ABT 1936 Determination of reduced ascorbic acid in small amounts of blood. Proc. Soc. Exp. Biol. Med., *34:* 146.

GIFFT, H. H., AND H. M. HAUCK 1946 A comparison of 4 methods for studying the urinary excretion of thiamine. J. Nutrition, *31:* 635.

HATHAWAY, M. L., AND J. E. STROM 1946 A comparison of thiamine synthesis and excretion in human subjects on synthetic and natural diets. J. Nutrition, *32:* 1.

KLOSTERMAN, A. M., J. E. HAINES, H. M. HAUCK AND A. B. KLINE 1947 The renal threshold for ascorbic acid. A modified method for its estimation with results for 12 adult subjects. J. Nutrition, in press.

LEWIS, J. S., C. A. STORVICK AND H. M. HAUCK 1943 Renal threshold for ascorbic acid in 12 normal adults with a note on the state of tissue reserves of subjects on an intake of ascorbic acid approximating the suggested daily allowance. J. Nutrition, *25:* 185.

LINGHORNE, W. J., W. G. MCINTOSH, J. W. TICE, F. F. TISDALL, J. F. MCCREARY, T. G. H. DRAKE, A. V. GREAVES AND W. M. JOHNSTONE 1946 The relation of ascorbic acid intake to gingivitis. Can. Med. Assoc. J., *54:* 106.

THE GROWTH OF THE ODONTOBLASTS OF THE INCISOR TOOTH AS A CRITERION OF THE VITAMIN C INTAKE OF THE GUINEA PIG[1]

E. W. CRAMPTON

Department of Nutrition, Macdonald College, McGill University, P.O., Prov. Quebec, Canada

FIVE FIGURES

(Received for publication November 22, 1946)

After describing briefly 2 physical methods, 21 chemical methods, and 1 biochemical method, Rosenberg ('45) stated "Although the chemical, and to a small extent also, physical methods are replacing more and more the biological determinations of vitamin C, the biological tests maintain their place as the ultimate and most correct method of determining vitamin C." The problem of the assay of this vitamin was of particular concern to the Canadian Government during the war years because of the difficulty of providing natural sources of vitamin C to the armed forces for a considerable portion of the year. Inasmuch as different chemical procedures frequently gave different results as to the potency of a food in which the armed forces were interested, this laboratory was requested in 1942 to undertake the establishment of a vitamin C bioassay which might be used as a check against chemical procedures.

THE BASAL DIET

Most biological assays for vitamins depend ultimately on the normal development of the experimental animal, either as

[1] Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Quebec, Canada. Journal Series no. 223.

a whole, as measured by growth; or of some tissue which can be observed separately and on which the vitamin appears to have specific effects. Since the development of the tissues of the body may be limited through diet inadequacies, it is obviously essential that any diet, to be fully satisfactory as the basis of a bioassay, must be nutritionally complete with the exception of the vitamin to be studied.

Insofar as we are aware, the nutritional requirements of the guinea pig are not as yet completely known — in the sense at least that it has not yet been possible to provide either a purified diet or one of natural foods which is fully successful in maintaining normal growth and reproduction, unless some fresh herbage is also fed. Several of the diets, fortified with ascorbic acid, which have been reported for the bioassay of vitamin C have proved in this laboratory to be entirely inadequate to support a successful pregnancy or to promote normal growth of young pigs to maturity.

We were faced at once, therefore, with the necessity of developing a basal diet for the assay. Suitable criteria of nutritional adequacy offered some problems, but due to the fact that the young guinea pig has a particularly long intra-uterine life, and that it is physiologically much more mature at birth than most other small animals, it was believed that dietary deficiencies might be manifest by reproductive failure or by sub-normal performance of breeding females confined to such rations. It was decided, therefore, to feed to pregnant guinea pigs, different basal diets suitable with respect to such factors as palatability, physical nature and freedom from vitamin C, and to note their ability to complete normal reproduction. Comparable females, fed the same diets plus green feed, were used as controls. All test basal diets were supplemented with ascorbic acid as the sole source of vitamin C.

In addition to the pregnancy tests, the more promising of the mixtures were subjected to growth-tests with groups of young animals fed against check groups receiving the same rations plus green feed.

As a result of these studies a basal diet has been devised for the vitamin C bioassay. It is called the Macdonald Guinea Pig Basal Diet no. 5, and has the following percentage composition: ground oats 15, ground wheat 13,[2] ground, dried beet-pulp 25, linseed oilmeal 12.5, skimmilk powder 15, fishmeal 5, brewer's dried yeast 10, bone char 4, and salt (iodized) 0.5.

For feeding, the mixture is pressed into pellets of about one-eighth inch diameter. We have found that while guinea pigs will scratch a meal mixture out of their feeders, they will eat the same mixture without waste when it is offered in small pellet form. In addition to the dry basal mixture, the pigs were supplied directly with: vitamins A and D as a feeding fish oil; vitamin E as alpha tocopherol; and vitamin C as ascorbic acid. It may be noted that wheat germ to the extent of 10% of the basal mixture did not prevent muscle degeneration and hemorrhage which were entirely corrected by daily allowances of 3 mg of alpha tocopherol.

In our experience, when this vitamin-supplemented ration is fed ad libitum along with either fresh or with dried, long-stored grass clippings, reproduction (80% successful pregnancies) and the growth of young are normal; whereas if the roughage is omitted, only about 66% of pregnancies are successful and there is some, though not a marked slowing of growth of the young to maturity. It may be noted that the dried herbage clippings used were not anti-scorbutic. Because of the evident craving for edible roughage, it is interesting to speculate as to whether or not some of the failure which has been reported in the preparation of purified diets for guinea pigs is not related to the need of this species for roughage material to maintain normal caecum function, comparable to the case of the rumen with ruminants.

From our observations of several hundred pregnant guinea pigs and of their progeny, we have now constructed what may

[2] Five pounds of this component may be replaced, if desired by an equal weight of molasses for greater ease in pelleting.

be called a "normal standard" for the behavior of guinea pigs on this diet regime. It may be summarized as in table 1.

Although it is evident from these tests that the diet which we are using without roughage for the odontoblast assay is not entirely complete nutritionally when judged by the severe criterion of adequacy for reproduction, yet young pigs carried on bioassay, on this diet, show no abnormalities at post-mortem examination at the end of the 42-day assay period provided the ascorbic acid has been supplied in amounts of 2 mg per pig per day or greater.

TABLE 1

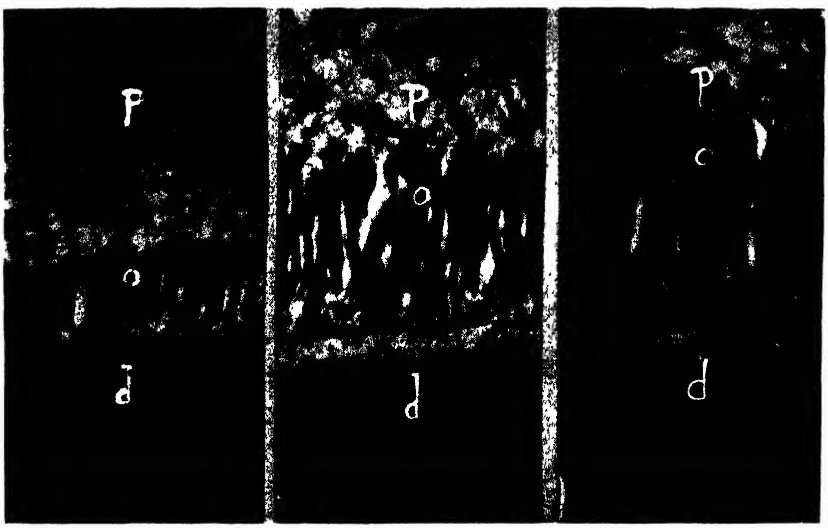
Typical average performance of guinea pigs on vitamins A, C, D and E supplemented Macdonald guinea pig diet no. 5.

RESPONSE WITH REGARD TO	WITHOUT ROUGHAGE	WITH FRESH OR DRIED ROUGHAGE
Successful pregnancies	67%	80%
Hemorrhage and resorption of fetus	20%	20%
Abortion	13%	0%
Number of pigs per litter:		
total	3.7	3.9
born alive	3.0	3.5
weaned	3.0	3.5
Birth weight of pigs born alive	100 gm	100 gm
Weight at 21 days (weaning time)	260 gm	275 gm
Gains per wk. from weaning time to 500 gm (females only)	30 gm	43 gm

The bioassay procedure

Numerous workers have described the radical changes which occur in the scorbutic guinea pig tooth and these changes have been used in subjective assay methods as an index of the vitamin C intake of young guinea pigs. All of these methods suffer from an inherent lack of precision. In 1940, an objective method of assay was proposed by Boyle, Bessey and Howe based on their finding that the width of the dentine layer of the incisor tooth varied with the vitamin C intake. This method

appeared to avoid the difficulty of assessing the degree of tooth damage involved in earlier tooth assay methods. However, in examining the teeth of pigs on varying intakes of ascorbic acid, we were impressed with the clear-cut and easily measured differences in the length of the odontoblast cells (see fig. 1).



0.5 mg 1.0 mg 2.0 mg

Fig. 1 Photomicrographs (× 440) showing development of the odontoblast cells in guinea pig incisor teeth resulting from the daily intake of 0.5, 1.0, and 2.0 mg of ascorbic acid, respectively. P, o, and d indicate pulp, odontoblasts and dentine, respectively.

In the mildly scorbutic guinea pigs the odontoblast cells at the formative end of the incisor tooth appear practically normal, while further incisally they become shorter and irregular in position (see fig. 2).

In the normal guinea pig, the odontoblasts, differentiating at the formative end of the tooth, increase in length until they reach maturity. At the senile end of the tooth, the odontoblasts have degenerated and are embedded in calcific scar tissue, or secondary dentine. Between these 2 well-defined

regions lies a row of tall columnar mature odontoblasts. Cell measurements must be taken in this area (figs. 3, 4 and 5).

These changes can be seen in longitudinal sections cut to expose the full length of the pulp cavity of the incisor tooth

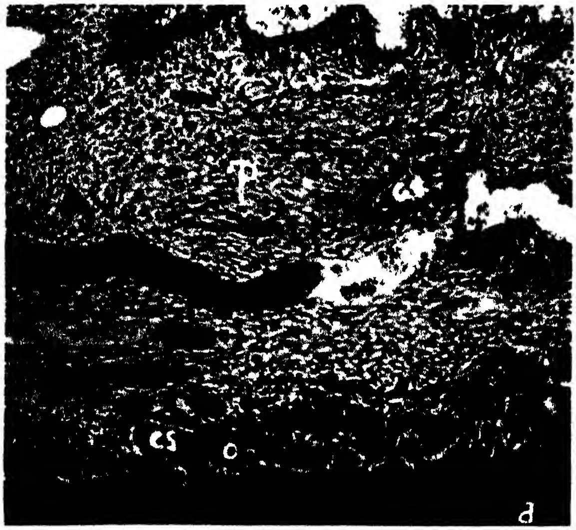


Fig. 2 Photomicrograph (× 100) showing irregular odontoblast row in a scorbutic tooth. (P) indicates disorganized pulp, (cs) calcific scar tissue, (o) degenerating odontoblasts, and (d) dentine, respectively.

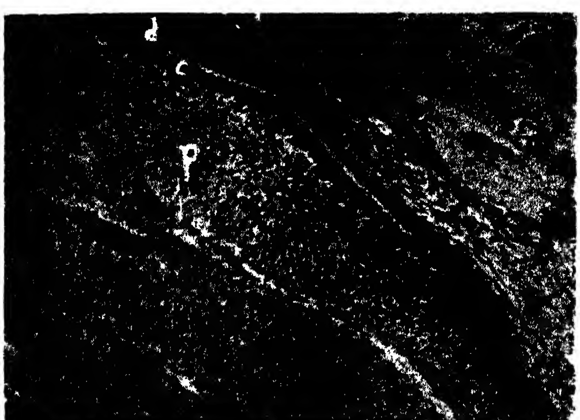


Fig. 3 Formative end of incisor tooth showing embryonic odontoblasts.

and are easily measured with a microscope fitted with a micrometer eye piece.

The length to which these cells develop in pigs of 250 to 400 gm weight, is apparently limited by the level of vitamin C intake, and ranges from about 30 microns with intakes of 0.5 mg of ascorbic acid daily, to a maximum of about 70 microns with intakes of 2 mg or over. Within this range of

Fig. 4 Mature odontoblast cells in area of reading.

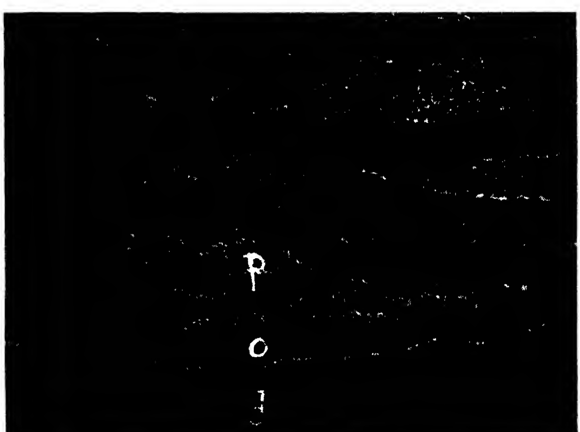


Fig. 5 Incisal end of incisor tooth showing senile odontoblasts. (P) indicates, pulp, (o) odontoblasts, and (d) dentine, respectively.

intake, the odontoblast length bears a logarithmic relation to the vitamin C fed. With intakes of less than 0.5 mg the cells are so disorganized that accurate readings are difficult, and in addition, frank scurvy is present in many individual animals which, in our opinion, renders them unacceptable for assay purposes.

Allotment considerations

We have now conducted tests involving some 650 pigs, aimed at establishing an assay procedure having the obviously desirable characteristics of precision, simplicity and reliability.

In this connection it may be stated that when raised to assay age on our vitamin supplemented basal diet, the absence or use of green feed is apparently immaterial. Further, there are no differences between the sexes in odontoblast cell development and the animals may be penned alone or in groups during the assay. Somewhat more regular curves have been obtained in assays of 35 and 42 days than on shorter periods, and selection of pigs for assay on the basis of age (28 days) has been preferable to the use of an attained live weight of 300 ($\pm$ 5) gm.

Limits of assay range

The response to ascorbic acid of pigs carried under assay conditions has been measured at daily intakes of 0.25, 0.50, 1.0, 2.0, 4.0, and 8 mg. In our experience, pigs carried for over 28 days on intakes of 0.25 mg of ascorbic acid have shown frank scurvy including capillary hemorrhage in the hind legs. No cases of clinical scurvy have been seen on intakes of 0.5 mg per day.

At the upper end of the range we have not found satisfactory increased response with intakes of 4.0 or 8.0 mg over that shown for 2.0 mg. We have not thus far investigated values intermediate between 0.25 and 0.5 mg, or between 2.0 and 4.0 mg of ascorbic acid.

Precision of the assay

In all tests, in addition to the odontoblast readings, the gains of the animals have been recorded and a gain response curve determined. When male animals only are used, the mean estimated potency of the carrier was frequently approximately the same as determined from the odontoblast readings. The response of the females, however, was usually unsatisfactory because of much slower gains.

The relative precision of the 2 methods (gain vs. odontoblast) is striking. The precision of an assay of this type may be expressed as the ratio of the standard error of the mean difference between 2 levels of vitamin intake and the slope of the regression connecting them,

$$pr = \frac{\sigma}{b} \times \sqrt{\frac{2}{n}} \qquad (1)$$

where n is the number in the group, σ the standard deviation of the assay, and pr the precision.

One measure of the relative precision may be indicated by the number of animals needed per group to give the same values for pr. For this purpose we may write the above formula in the form of

$$n = \frac{2}{pr^2} \times \frac{\sigma^2}{b^2} \qquad (2)$$

The number of animals needed per group (i.e., test level group) where growth is the criterion to give a precision *(pr)* equal to that attained with the number actually used for the odontoblast readings may then be calculated by using in formula (2) the values of pr^2 found for the odontoblast readings and those of σ^2 and b^2 found for the growth values. The n thus found will be compared to the n used in the odontoblast calculations (see column "nc" of table 2).

Another way of indicating relative precision involves a comparison of the precision indexes of the 2 methods. This is shown in the last column of table 2.

The relevant data for 7 separate assays are given in table 2. In our experience the odontoblast method has consistently given an appreciable increase in precision over the growth method of estimating vitamin C intake.

TABLE 2

Relative precision of growth vs. odontoblasts as criteria of vitamin C intake for guinea pigs.

SUBSTANCE ASSAYED	ODONTOBLAST METHOD				GROWTH METHOD				nc [1]	RELATIVE PRECISION
	σ	b	n	pr	σ'	b'	n'	pr'		
Aqueous soln. ascorbic	2.8	32	10	.039	49.3	247	10	.089	53	.44 [2]
Fresh orange juice	3.5	21	10	.074	56.5	61	10	.415	314	.18
Conc. orange juice	5.4	59	6	.053	26.5	135	6	.113	28	.47
Aqueous soln. ascorbic	3.2	21	10	.070	57.6	105	10	.244	122	.29
Fresh orange juice	3.2	18	10	.082	57.6	122	10	.211	66	.39
Synthetic orange juice	3.2	22	10	.065	57.6	94	10	.273	177	.24
Apple juice	3.2	33	10	.044	57.6	192	10	.134	95	.33

[1] *nc* in growth assay to give *pr* equal to odontoblast assay.

[2] *i.e.*, Growth assay has 44% the precision of the odontoblast assay.

Details of assay procedure

The details of the procedure now used in this laboratory are as follows:

Young guinea pigs bred in our own colony and raised from mothers maintained on the basal diet, above described, supplemented with vitamins A, C, D and E plus either hay or greenfeed as available, are at 28 (± 3) days of age, allotted at random to individual cages and allowed free access to the pelleted basal diet and water. Vitamins A, D and E in corn oil are administered weekly, by means of a calibrated hypodermic syringe, with needle removed, in amounts to provide the equivalent of 425 I.U. of vitamin A, 48 I.U. of vitamin D and 3 mg of alpha tocopherol daily.

Both the unknown and the control source of vitamin C are fed at each of at least 3 levels of intake, spaced at equal log intervals. We have continued the restriction in allotment that there shall be equal numbers of each sex on each dose level,

and for convenience, have continued the use of individual penning. (Normally not less than 10 animals per dose are used.)

The feeding period for all pigs is 42 days. Since individual penning is employed, different pigs can start and hence complete a trial at different times. This is advantageous not only in the problem of obtaining the needed numbers of animals for assay at any given time, but especially in avoiding the necessity for processing undue numbers on a single day at the end of the feeding period.

Preliminary preparation of the tooth

At the conclusion of its 42-day feeding period each animal is sacrificed by chloroform. Its lower jaw is removed and divided by a vertical incision between the incisors. The exposed portions of the incisor and that portion of the mandible extending beyond the molars are clipped off (thus allowing the fixing agent more easily to reach the pulp of the incisor), and the remainder placed in 10% formalin.

Following a minimum fixation period of 48 hours in 10% formalin, the teeth are washed in 70% alcohol for 24 hours. Decalcification [3] is then carried out in 10% nitric acid, changing the acid every second day. In 36 to 48 hours the unwanted molar and a jaw tissue is readily trimmed away. The tooth is tested with a sharp needle and is removed from the acid when the entire specimen can be pierced easily. Complete decalcification is usually accomplished in 3 to 4 days.

After rinsing in 1 or 2 changes of water, the teeth are placed in 2% potassium alum for 12 hours. This is followed by another rinse in water and a transfer to 5% sodium bicarbonate for 24 hours.

The incisors are then thoroughly washed in running water for 12 to 24 hours, using a washing bobber, and are prepared for embedding in the steps indicated in table 3.

[3] Based on method published by F. W. Gairns. Stain Technology, vol. 19, no. 4, 1944.

The teeth are now orientated for longitudinal sectioning, embedded in new 60°C. paraffin, and cooled.

Cutting. The paraffin block containing the embedded tooth is secured in the microtome jaw and sections removed until the center of the tooth is exposed, i.e., to the point where the pulp cavity ceases to increase in length or width. From the ribbon of sections representing the tooth center, 4 to 6 sections of 8 to 10 microns in thickness are selected for microscopic examination.

TABLE 3

Steps through alcohol to paraffin.

MATERIAL	IN	OUT
10% alcohol [1]	9:00 a.m.	12:00 noon
20% alcohol [1]	12:00 noon	5:00 p.m.
40% alcohol [1]	5:00 p.m.	9:00 a.m.
60% alcohol [1]	9:00 a.m.	12:00 noon
80% alcohol [1]	12:00 noon	5:00 p.m.
Absolute alcohol	5:00 p.m.	9:00 a.m.
Absolute alcohol	9:00 a.m.	10:30 a.m.
Cedarwood oil	10:30 a.m.	12:00 noon
52°C. paraffin	12:00 noon	2:30 p.m.
60°C. paraffin	2:30 p.m.	4:30 p.m.
	changing once	

[1] Phenol may be added to these alcohols up to 6% to impart an elasticity to the tissues, thus facilitating the cutting of thin sections.

Chilling both knife and blocks in ice water for 10 minutes before sectioning increases the ease with which sections are cut.

Staining. Erhlich's acid haematoxylin and the counterstain eosin are employed in ordinary progressive staining.

The odontoblast row of each of the sections on the slide is examined and 5 readings are taken from that section bearing the highest odontoblasts. The average of these is considered to represent the maximum height of the odontoblast cells of that animal.

Measurements are made under 440 magnification by means of an ocular micrometer and the readings are subsequently

converted to microns. Groups of cells of the same height in the central area of maximum odontoblast development are chosen. Measurements are not made in the regions of the dental papilla, nor at the biting end of the tooth where the odontoblasts are seen in their embryonic and senile states, respectively. On levels of ascorbic acid below 1.0 mg the odontoblast development is more irregular and the avoidance of single cell readings is not always possible.

Statistical treatment of the data

The feeding tests are designed to permit standard analysis of the variance of the data and the application of the factorial scheme for isolating individual treatment effects as described by Bliss and Marks ('39). As already mentioned, at least 3 levels of known and unknown are fed. The factorial analysis, however, is applied to the levels of unknown which lie within the limits of the linear response of the known. By this procedure, the difficulty with levels of unknowns which prove to lie outside the limits of linear response is avoided.

ACKNOWLEDGMENTS

The series of studies upon which this report is based was made possible through the generous financial assistance of the Department of Pensions and National Health, now the Department of National Health and Welfare of the Canadian Government. The tedious work involved in the conduct of the numerous assays and especially in the histology, was carried in turn by Barbara Collier, Dr. L. D. Woolsey, Muriel Hughes, Florence Farmer, Milton Bell and Barbara Burton, former graduate students in this Department. Their assistance is gratefully acknowledged.

LITERATURE CITED

BLISS, C. I., AND H. P. MARKS 1939 The biological assay of insulin. II. The estimation of drug potency from a graded response. Quart. J. Pharm. and Pharmacol., *12*: 182.

Boyle, P. E., O. A. Bessey and P. R. Howe 1940 Rate of dentine formation in incisor teeth of guinea pigs on normal and on ascorbic-acid-deficient diets. Arch. Path., *30:* 90.

Rosenberg, H. R. 1945 Chemistry and Physiology of the Vitamins. Interscience Publishers, Inc., N. Y.

THE RENAL THRESHOLD FOR ASCORBIC ACID

A MODIFIED METHOD FOR ITS ESTIMATION WITH RESULTS FOR TWELVE ADULT SUBJECTS [1]

ANN MOORE KLOSTERMAN, J. ESTELLE HAINES,
HAZEL M. HAUCK AND ALICE B. KLINE

*New York State College of Home Economics, and School of Nutrition,
Cornell University, Ithaca, New York*

(Received for publication November 27, 1946)

A method for estimating the renal threshold for ascorbic acid has been reported from this laboratory by Lewis, Storvick and Hauck ('43). A majority of their subjects were studied for 2-week periods, with daily determinations of fasting plasma ascorbic acid and corresponding 1-hour urinary excretion values. In later studies in this laboratory, such determinations were made daily for 6 weeks. With a large number of fasting values, a statistically significant difference was sometimes found between 2 means, both of which were below the renal threshold as estimated by inspection of the arrayed data and scatter diagrams. This paper presents a modified method for treating such data, with values for renal thresholds for ascorbic acid for 12 adult subjects, 1 of whom was previously studied by Lewis, Storvick and Hauck ('43).

EXPERIMENTAL PROCEDURE

Personal data concerning the subjects, the diets and experimental procedure are reported in connection with the studies

[1] Some of the data in this paper are taken from theses presented to the faculty of the Graduate School of Cornell University in fulfillment of the requirement for the M.S. degree, by Ann C. Moore, June 1944, and J. Estelle Haines, February 1946. Further details will be found in the theses.

on tissue reserves of ascorbic acid at various intakes (Haines et al., '47).

Blood and urine samples to be used in estimating the renal threshold were obtained as described by Lewis, Storvick and Hauck ('43) for their second series of experiments, i.e., the blood sample was taken midway in the hour for which the urine specimen was collected. Some of these specimens were obtained under fasting conditions, and some after various sized doses of ascorbic acid.

METHOD OF ESTIMATING THE RENAL THRESHOLD

In our modified method, inspection of the means and standard deviations for hourly urinary excretion of ascorbic acid at various plasma levels is substituted for Student's ('25) "t" test for determining the mathematical significance of the differences between means. The first step was to array the data for plasma ascorbic acid values for each subject, with the corresponding hourly urinary excretion values. Scatter diagrams were also made from these data. From inspection the apparent renal threshold for ascorbic acid was noted for each subject. The mean value, with its standard deviation, for urinary excretion of ascorbic acid at the apparent renal threshold was then calculated. This was compared with the mean value and its standard deviation for urinary excretions at all plasma levels below the apparent renal threshold. Since by definition, the renal threshold for ascorbic acid is the plasma concentration at which a marked rise in urinary excretion of ascorbic acid occurs, if the threshold value obtained by inspection is correct, the excretion of ascorbic acid at this plasma value will be relatively large and variable. Below the renal threshold, the excretion is low and relatively constant (Friedman, Sherry and Ralli, '40). To test the validity of the apparent renal threshold, similar calculations were made for plasma values 0.1 mg % below and 0.1 mg % above the apparent renal threshold.

RESULTS AND DISCUSSION

Renal threshold values for 12 subjects

Mean urinary excretion values for ascorbic acid corresponding to plasma values at, above and below the renal thresholds for 12 subjects are presented in table 1. For these subjects, the renal threshold values for plasma ascorbic acid as estimated by this method, ranged from 1.0 to 1.3 mg %. Mean excretion values for ascorbic acid at the threshold were all above 2 mg per hour, with most standard deviations considerably more than half the mean.

Of the renal thresholds for ascorbic acid reported in this paper and that of Lewis, Storvick and Hauck ('43), for 23 individuals, 21 were between 1.0 and 1.3 mg %. Inspection of the data for the entire series suggests that if a subject excretes 2 mg or more of ascorbic acid in 1 hour, his plasma ascorbic acid value has almost certainly exceeded his renal threshold during that hour, whereas if the excretion is 1.0 mg or less, it probably has not.

Comparison of the modified method for estimating renal threshold for ascorbic acid with that of Lewis, Storvick and Hauck ('43)

When the method described in this paper was applied to the data of Lewis, Storvick and Hauck ('43), the estimated renal thresholds were the same in all cases. When the method of Lewis, Storvick and Hauck was applied to data for the subjects of the present study, odds for significance for points below the apparent renal threshold were more comparable with those of Lewis, Storvick and Hauck when the fasting plasma values used in the calculations were limited to 2 weeks of the study. Thus the method presented here appears to have a wider usefulness than that previously devised in this laboratory.

Stability of the renal threshold for ascorbic acid

In the present study, 4 subjects, E.K., D.L., H.G. and A.M., were subjects for 2 successive years. Subject J.M. served

TABLE 1

Mean urinary excretion values for ascorbic acid corresponding to plasma values at, above, and below the renal threshold for ascorbic acid. (12 subjects.)[1]

SUBJECT	RENAL THRESHOLD	HOURLY EXCRETION AT THRESHOLD	HOURLY EXCRETION BELOW THRESHOLD	PLASMA VALUE BELOW THRESHOLD	HOURLY EXCRETION AROUND POINT TESTED	HOURLY EXCRETION BELOW POINT TESTED	PLASMA VALUE ABOVE THRESHOLD	HOURLY EXCRETION AROUND POINT TESTED	HOURLY EXCRETION BELOW POINT TESTED
	mg %	mg	mg	mg %	mg	mg	mg %	mg	mg
M. H.[2]	1.0	2.6 ± 1.3 (5)[3]	0.7 ± 0.4 (7)	0.9	1.4 ± 0.6 (2)	0.6 ± 0.2 (15)	1.1	2.4 ± 0.8 (5)	1.1 ± 1.1 (22)
J.L.	1.0	3.3 ± 1.9 (4)	1.1 ± 0.6 (38)	0.9	1.7 ± 0.7 (6)	1.0 ± 0.5 (32)	1.1	3.1 ± 1.9 (3)	1.2 ± 0.9 (41)
P.H.	1.05	5.7 ± 7.6 (4)	0.9 ± 0.4 (38)	0.95	1.3 ± 0.7 (5)	0.8 ± 0.3 (33)	1.15	7.8 ± 7.1 (4)	1.3 ± 2.5 (42)
E.K.	1.1	2.8 ± 2.0 (5)	1.4 ± 0.6 (46)	1.0	1.9 ± 0.8 (6)	1.3 ± 0.5 (40)	1.2	2.8 ± 1.5 (7)	1.3 ± 0.6 (46)
H.G.	1.1	2.1 ± 1.6 (7)	1.0 ± 0.3 (24)	0.95	1.3 ± 0.4 (3)	1.0 ± 0.2 (21)	1.25	3.4 ± 3.5 (6)	1.2 ± 0.9 (31)
J.M.[4]	1.1	3.2 ± 4.3 (9)	0.9 ± 0.5 (32)	1.0	0.9 ± 0.3 (6)	0.9 ± 0.5 (29)	1.2	8.3 ± 6.3 (5)	0.9 ± 0.4 (37)
D.L.[2]	1.15	4.2 ± 3.7 (5)	0.8 ± 0.3 (38)	1.05	1.3 ± 0.6 (3)	0.8 ± 0.2 (35)	1.25	2.7 (1)	1.2 ± 1.5 (43)
A.M.	1.2	7.7 ± 7.1 (3)	0.8 ± 0.4 (43)	1.1	1.2 ± 0.8 (6)	0.7 ± 0.2 (37)	1.3	4.8 ± 5.7 (6)	1.2 ± 2.3 (46)
L.G.	1.3	6.5 ± 4.3 (7)	1.0 ± 0.4 (27)	1.2	1.2 (1)	1.0 ± 0.4 (26)	1.4	6.3 ± 0.1 (2)	2.2 ± 3.0 (34)
J.E.S.[2]	1.3	2.8 ± 1.6 (3)	1.1 ± 0.5 (21)	1.2	1.9 ± 0.2 (2)	1.0 ± 0.4 (19)	1.4	2.5 ± 1.7 (6)	1.3 ± 0.9 (24)
E.H.	1.3	3.3 ± 2.3 (8)	1.0 ± 0.5 (28)	1.2	1.1 ± 0.4 (4)	1.0 ± 0.6 (24)	1.4	6.8 ± 1.2 (4)	1.5 ± 1.5 (36)
S.P.	1.3	2.2 (1)	0.9 ± 0.3 (30)	1.2	1.1 ± 0.2 (4)	0.8 ± 0.3 (26)	1.4	4.8 ± 0.2 (2)	0.9 ± 0.4 (41)

[1] When a subject was studied during 2 successive years, values for the larger series of the 2 are given.
[2] Some of the determinations used in calculating the renal threshold for these subjects were made by Margaret Delaney.
[3] Figures in parentheses represent number of values included in the mean.
[4] This subject also served for the experiments of Lewis, Storvick and Hauck ('43).

for this experiment and also that of Lewis, Storvick and Hauck ('43). These authors also reported the renal threshold for 1 subject (J.S.) who served for 2 successive years. Whether values for these subjects were calculated for the separate years or combined, the estimated renal thresholds were the same. Evidence from these 6 subjects suggests, therefore, that the renal threshold for ascorbic acid is relatively stable in the normal adult from year to year, and, within the limits of this experiment, at various levels of intake.

SUMMARY

A modified method for estimating the renal threshold for ascorbic acid based on the comparison of means and standard deviations for urinary excretions at various plasma ascorbic acid levels is presented. This method is simpler and appears to have a wider usefulness than that previously reported from this laboratory.

The renal thresholds for 12 normal adults, as estimated by this method, were between 1.0 and 1.3 mg %.

Further evidence is presented that the renal threshold for ascorbic acid is relatively stable from year to year.

LITERATURE CITED

FRIEDMAN, G. J., S. SHERRY AND E. P. RALLI 1940 The mechanism of the excretion of vitamin C by the human kidney at low and normal plasma levels of ascorbic acid. J. Clin. Inv., *19:* 685.

HAINES, J. E., A. M. KLOSTERMAN, H. M. HAUCK, M. A. DELANEY AND A. B. KLINE 1947 Tissue reserves of ascorbic acid in normal adults on 3 levels of intake. J. Nutrition, *33:* 479.

LEWIS, J. S., C. A. STORVICK AND H. M. HAUCK 1943 Renal threshold for ascorbic acid in 12 normal adults with a note on the state of tissue reserves of subjects on an intake of ascorbic acid approximating the suggested daily allowance. J. Nutrition, *25:* 185.

STUDENT 1925 New tables for testing the significance of observations. Metron, *5:* 105.

PLASMA LEVELS AND URINARY EXCRETION OF ASCORBIC ACID IN WOMEN DURING THE MENSTRUAL CYCLE [1]

HAZEL M. HAUCK
New York State College of Home Economics, and School of Nutrition, Cornell University, Ithaca, New York

(Received for publication November 27, 1946)

Variations in the urinary excretion of ascorbic acid (Fernandes, '39; Pillay, '40; Mickelson et al., '43; Hartzler, '45) and in fasting plasma ascorbic acid values (Mickelson et al., '43) in relation to the menstrual cycle, have been reported, but these reports are not in agreement. Fernandez ('39) observed "inconceivably high values" for dye reduction by urine just before menstruation, whereas Hartzler noted low values for urinary excretion of ascorbic acid just before and/or during the early part of the menstrual period. Pillay ('40) found the lowest excretion of ascorbic acid after a test dose at the mid-point of the menstrual cycle, whereas Mickelson et al. ('43) found "a sharp increase in the fasting level of plasma vitamin C" in some women during the middle of the menstrual cycle, with some increase in vitamin C excretion on the days corresponding to this peak.

Some day-to-day variations occur in fasting plasma levels and urinary excretion of vitamin C even on a constant diet. Of the investigations mentioned, however, only 2 were during controlled vitamin C intake, namely that of Hartzler ('45) in which the variations with menstruation were noted in 1 series

[1] Most of the vitamin C determinations were made by Clara A. Storvick, Ann C. Moore, Alice Kline, Margaret Delaney and Estelle Haines in connection with Purnell projects directed by the author.

of experiments but not in another, and that of Mickelson et al. ('43) in which 1 of 8 subjects was kept on a constant vitamin C intake throughout 1 menstrual cycle.

Since, in the course of our studies on vitamin C metabolism, a number of women subjects have been maintained on a constant level of vitamin C intake for periods of 4 and 6 weeks, the data obtained on fasting plasma values and urinary excretion of vitamin C were examined to see if any relation to the menstrual cycle could be noted.

EXPERIMENTAL

Ten women subjects served during experimental periods of 4 or 6 weeks, i.e., enough to include 1 menstrual cycle. In all, data were available for 30 menstrual periods. In 10 instances 2 menstrual periods occurred in the course of an experiment, so that the urinary excretion and the plasma ascorbic acid values could be noted for the midpoint of the cycle.

Details as to the experimental procedures and diet are reported elsewhere (Belser, Hauck and Storvick, '39; Storvick and Hauck, '42; Haines et al., '47). The results are presented in table 1. When a menstrual period occurred at the beginning or end of an experiment, values for those days during the presaturation or resaturation periods were not included, i.e., all values shown in table 1 are for days or periods on the constant level of intake stated.

Since all of the experiments began with a period of high intake of ascorbic acid to assure saturation, there was some downward trend in urinary excretion and plasma ascorbic acid values for all subsequent intakes. The week of the experiment during which the menstrual period occurred is indicated since, in general, weekly means for the early part of the experiment were above the general mean, whereas those toward the end of the experiment were below it. Most instances in which the values related to the menstrual period differed from the mean by more than the standard deviation occurred toward the beginning or end of the experiment, and this circumstance, rather than the associated menstrual period,

TABLE 1

Plasma values and urinary excretion of ascorbic acid in relation to the menstrual period.

SUBJECT	LEVEL OF INTAKE, MG	WEEK [1]	ASCORBIC ACID IN URINE, MG: Mean for expt.[2]	Day before M.P.	First day of M.P.	Mean for M.P.	Mid-value [3]	Week of mid-value [4]	PLASMA ASCORBIC ACID VALUES, MG %: Mean for expt.[2]	Mid-value [3]
D.L.	53	3	23 ± 6	22	22	22				
	50	3	18 ± 4	16	18	18				
J.E.S.	50	2	21 ± 4	25	22	19	17	4	0.9 ± 0.2	0.8
	50	6	21 ± 4	*15*	*16*	*16*				
M.H.	50	3	18 ± 5	20	16	17	17	4	0.7 ± 0.1	0.6
	50	6	18 ± 5	15	16	[5]				
E.H.	33	4	25 ± 4	23	*30*	26	26	2	0.5 ± 0.2	0.7
L.G.	33	3	19 ± 3	21	20	21	18	5	0.5 ± 0.2	0.4
A.M.	70	3	24 ± 6	20	21	22	18	4	0.9 ± 0.1	0.8
	70	6	24 ± 6	18	26	23 [6]				
	53	4	19 ± 3	18	18	18	20	2	0.8 ± 0.2	0.9
C.S.	210	3	154 ± 18	148	140	146				
	135	1	82 ± 10	90	*97*	85				
	110	1	59 ± 10	68	64	62				
	100	3	49 ± 20	30	31	35				
	65	2	29 ± 8	26	29	26				
K.J.	210	2	164 ± 14	153	171	162				
	85	1	45 ± 10	*75*	*64*	*59*	37	2	1.2 ± 0.1	1.1
	85	4	45 ± 10	35	40	[5]				
	75	3	38 ± 11	44	35	38				
H.H.	210	3	163 ± 13	165	*144*	160				
	135	2	87 ± 11	93	[7]	96 [7]				
	110	3	65 ± 12	70	61	64				
	100	1	58 ± 13	*162*	*110*	*90*	50	2	0.8 ± 0.1	*0.6*
	100	4	58 ± 13	58	52	[5]				
	60	2	26 ± 11	26	30	25				
J.S.	210	1	158 ± 14	*226*	*175*	*184*	154	2	1.3 ± 0.1	1.3
	210	4	158 ± 14	164	151	149				
	60	1	26 ± 9	[8]	*170*	*89*	30	2	1.0 ± 0.1	1.0
	60	4	26 ± 9	21	21	20 [6]				

[1] Week of experiment in which menstrual period started.
[2] Values for first 2 days of experimental period omitted in calculating the general mean.
[3] Value for mid-point of menstrual cycle.
[4] Week of experiment in which mid-point of menstrual cycle occurred.
[5] One day only on constant intake.
[6] Two days only on constant intake.
[7] Value not obtained on first day of menstrual period.
[8] During presaturation period.

Values which differ from the mean value for the period by more than the standard deviation have been italicized.

might account for the deviation from the mean value for the period. Note, for example, that of 30 urinary excretion values, obtained on the day before a menstrual period only 4 differed from the mean value by more than the standard deviation. The 3 instances in which the differences were positive, all occurred during the first week of the experiment, whereas the 1 instance in which the difference was negative occurred in the last week of the experiment. Similarly, few marked variations from the means were noted among urinary excretion values for the first day or for the entire menstrual period. In most of these instances, the downward trend in urinary excretion values for vitamin C, rather than menstruation, might account for the unusual values.

Of 10 plasma ascorbic acid values obtained at the mid-menstrual period for 8 women, only 1 differed from the mean for the experiment by more than the standard deviation. In no instance was there an unusual ascorbic acid excretion at the mid-menstrual period.

Not only were unusual variations in urinary excretion of ascorbic acid at the menstrual period the exception rather than the rule, but such unusual variations as were noted were not consistently in 1 direction. Equally great variations, both positive and negative, have been noted in excretion values for our male subjects.

SUMMARY

In 10 women subjects whose ascorbic acid metabolism was studied for a total of 23 periods of from 4 to 6 weeks duration, during which 30 menstrual periods occurred, no evidence was found of unusual variability in either urinary excretion of ascorbic acid or fasting plasma ascorbic acid values associated with the menstrual cycle.

LITERATURE CITED

BELSER, W. B., H. M. HAUCK AND C. A. STORVICK 1939 A study of the ascorbic acid intake required to maintain tissue saturation in normal adults. J. Nutrition, *17*: 513.

FERNANDES, A. 1939 The excretion of vitamin C in urine and the state of saturation in normal individuals in Bombay. J. Univ. Bombay, *7:* 111.

HAINES, J. E., A. M. KLOSTERMAN, H. M. HAUCK, M. A. DELANEY AND A. B. KLINE 1947 Tissue reserves of ascorbic acid in normal adults on 3 levels of intake. J. Nutrition, *33:* 479.

HARTZLER, E. R. 1945 The availability of ascorbic acid in papayas and guavas. J. Nutrition, *30:* 355.

MICKELSEN, O., A. L. DIPPEL AND R. L. TODD 1943 Plasma vitamin C levels in women during the menstrual cycle. J. Clin. Endocrinology, *3:* 600.

PILLAY, A. P. 1940 Vitamin C and ovulation. Indian Med. Gaz., *75:* 91.

STORVICK, C. A., AND H. M. HAUCK 1942 Effect of controlled ascorbic acid ingestion upon urinary excretion and plasma concentration of ascorbic acid in normal adults. J. Nutrition, *23:* 111.

BIOTIN BALANCE IN THE ALBINO RAT[1]

MARGARET A. McGREGOR, HELEN T. PARSONS AND
WILLIAM H. PETERSON

Departments of Home Economics, and Biochemistry, University of Wisconsin, Madison

(Received for publication July 15, 1946)

The theory that "avidin" (Eakin et al., '40, '41) or "antibiotin" (Woolley and Longsworth, '42) of raw egg-white combines with biotin in the digestive tract making biotin unavailable for absorption and resulting in an induced deficiency (Parsons et al., '40; György et al., '41) is now well accepted. Du Vigneaud et al. ('42) have suggested that the urea grouping of biotin is essential for the combination of biotin with avidin. The present study was initiated in an attempt to obtain additional evidence on the biotin-avidin interrelationship in the digestive tract by means of biotin balances. The experimental data were obtained during the years 1940–1942 but because of unforseen circumstances publication has been delayed until the present time.

EXPERIMENTAL

The fate of the biotin in the presence and absence of egg-white was studied in young rats during induced deficiency and recovery periods, and in adult rats on rations with and without egg-white and a rich source of biotin.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

Rations

Rations containing either raw or thoroughly cooked dried egg-white were used in this study. Table 1 shows the levels at which the egg-white was included in the various rations. In 1 series (lots I, II, III, VII, VIII and IX), the other ingredients were (Osborne and Mendel, '19) salt mixture 4%, dried yeast 5%, wheat germ (or rice polishings) 10%, lard 5%, sugar (or starch) to make 100%. In another series (lots IV, V, VI, XI and XII) cooked pork kidney replaced the yeast, lard and wheat germ (or rice polishings) and the sugar or starch was adjusted accordingly. A third kind of ration (stock) consisting of casein 5%, dried yeast 3%, wheat germ 2.5%, yellow corn 50%, oil meal 11%, alfalfa meal 1.5%, butterfat 5%, cod liver oil 2.5%, $CaHPO_4 \cdot 2H_2O$, 1%, NaCl 0.5%, and dried milk 18% was fed to lots X and XIII.

Animals

Albino rats were prepared for the experiment by placing them at weaning time on a ration containing 20% dried raw egg-white. When they had regained their weaning weight, the amount of raw egg-white in the diet was increased to 40% and finally to 66%. Moderate to severe biotin deficiency manifestations appeared in about 50 days. The manifestations of the induced biotin deficiency on these rations have been described (Parsons, '31; Parsons et al., '37). Inasmuch as the deficiency was induced in rats on rations containing 20% of egg-white as well as on 66%, the higher protein concentration presents no important complication in an interpretation of the results. At this time, the animals were placed in metabolism cages and urine and feces collections were made over a 4-day period (lot I, table 1). To study further the biotin balance during the deficiency period, the rats were then divided at random into 3 groups. One group was continued on 66% raw egg-white, a second was placed on 30% raw dried egg-white (lot II) and the third was fed 30% cooked dried egg-white (lot III). After 15 to 25 days had elapsed, urine and

feces were collected from lots II and III. Following this, some of the animals of the 3 groups just mentioned were chosen at random, and cooked dried pork kidney, an excellent, natural source of biotin,[2] was incorporated in their ration at a level of 25%. These groups are designated as lots IV, V and VI, and were taken from lots I, II and III, respectively. Collections were made of the elimination products after a 7- to 10-day adjustment period.

It was thought that a group of adult animals with similar nutritional histories on egg-white rations would be valuable in showing the effect of high levels of egg-white in the diet on the fecal output even though urinary studies could not be included. Such a group of rats which had been kept on a 20% raw egg-white ration and which had reached a fairly constant level of biotin elimination was divided into 3 groups. One group was continued on the 20% raw dried egg-white ration (lot VII), the second group was placed on a ration containing 66% raw dried egg-white (lot VIII) and the third on 66% cooked dried egg-white (lot IX).

Adult rats on the stock ration (lot X) were used to indicate the biotin balance of animals on an egg-white-free ration.

In an attempt to find the response of normal rats to the presence of egg-white and biotin in the ration, 2 of the rats from lot X were placed on a ration containing 30% raw dried egg-white and 25% cooked pork kidney (lot XI), and 2 were fed a ration containing 30% cooked dried egg-white plus 25% cooked dried pork kidney (lot XII).

The biotin balances of the stock rats and of the rats on the various egg-white rations are summarized in table 1.

On completion of the collections of urine and feces, the animals were killed. The livers and kidneys were removed and analyzed to determine if the biotin content of these organs bore any relationship to biotin intake or output. The contents of the digestive tract — the stomach, small intestine, cecum,

[2] Pure biotin would have been preferable for this purpose, but, unfortunately, at the time the work was done (1940–1941) adequate supplies of crystalline material were not available.

and large intestine — were also assayed for biotin. Such results might give some indication of the site of avidin-biotin fixation, and provide evidence for or against biotin synthesis in the digestive tract with the possible location of that synthesis. Only 3 of the animals in each of lots VII and VIII were used for this part of the experiment. These animals form lots VIIa and VIIIa given in table 2. Lot VIa is made up of the animals in lot VI, table 1, and 1 additional rat of the same nutritional history. Lot XIII was composed of a group of adult rats on the stock ration. This was a separate group from lot X.

Assays

Urine and feces were collected separately, daily. The feces were dried at room temperature; the urine was preserved with toluene and chloroform and kept in the refrigerator. At the end of the 4-day period, composite samples were made.

Biotin was determined by the microbiological assays of Lampen et al. ('41) and of Snell et al. ('40). All biotin values are based on at least 3 levels of assay in which results agreed within 10%.

As biotin in urine is present in water-soluble form no further treatment of the urine samples was required. Feces, liver and kidney, and contents of the digestive tract, however, contain bound biotin and this was liberated by acid hydrolysis. One gm of the dry substance was suspended in 40 ml of 2 N H_2SO_4, autoclaved for 2 hours at 15 lbs. pressure, cooled, neutralized with NaOH, filtered, and made up to 50 ml. If not assayed immediately, the hydrolyzed samples were preserved with toluene and chloroform and stored in the cold room until assayed.

RESULTS AND DISCUSSION

Biotin balances of rats on stock ration

Normal adult rats on the stock ration (table 1, lot X) eliminated from 0.7 to 2.0 μg (average 1.1 μg) of biotin daily in the feces while the output in the urine ranged from 0.6 to 1.0 μg (average 0.8 μg). Thus there was an approximately equal

TABLE 1

Biotin balances of albino rats on stock and egg-white rations.

LOT	INGREDIENTS OF RATION		NUMBER OF RATS	AVERAGE DAILY FOOD INTAKE	AVERAGE DAILY BIOTIN INTAKE [2]	AVERAGE DAILY ELIMINATION OF BIOTIN [2,3]			RATIO OF AV. BIOTIN ELIMINATION TO INTAKE
	Kind [1]	Amount				Urine	Feces	Total	
		%		gm	μg	μg	μg	μg	
I	EWR	66	10	5.5	3.2	0.6	4.4	5.0	
					(2.6–3.7)	(0.1–1.2)	(2.8–6.1)	(2.8–7.3)	1.6
II	EWR	30	3	7.7	2.3	0.6	4.6	5.2	
					(2.1–2.4)	(0.5–0.8)	(2.7–8.0)	(3.3–8.5)	2.2
III	EWC	30	4	11.5	3.7	0.8	4.8	5.6	
					(3.2–4.1)	(0.7–1.2)	(2.9–5.5)	(3.6–6.7)	1.5
IV	EWR	66			15.3	1.1	23.6	24.7	
	PKC	25	3	13	(14.2–17.5)	(1.0–1.1)	(21.8–25.2)	(22.3–26.4)	1.6
V	EWR	30	2	16	16.9	3.2	14.3	17.5	
	PKC	25			(15.8–17.9)	(3.0–3.4)	(13.2–15.4)	(16.6–18.4)	1.0
VI	EWC	30	2	10	10.5	3.2	4.6	7.8	
	PKC	25			(9.5–11.6)	(3.0–3.3)	(3.1–6.1)	(6.1–9.4)	0.7
VII	EWR	20	11	6.0	1.6		3.2		
					(1.1–2.6)		(1.6–5.3)		
VIII	EWR	66	5	4.8	3.0		4.2		
					(1.2–3.8)		(1.3–3.8)		
IX	EWC	66	2	8.5	5.3		5.8		
					(5.0–5.6)		(5.0–6.7)		
X	Stock	100	4	9.8	1.7	0.8	1.1	1.9	
					(1.3–3.0)	(0.6–1.0)	(0.7–2.0)	(1.3–3.0)	1.1
XI	EWR	30	2	8	8.5	2.2	10.1	12.3	
	PKC	25			(7.4–9.5)	(2.0–2.4)	(9.9–10.3)	(12.0–12.7)	1.4
XII	EWC	30	2	8	8.5	2.6	4.4	7.0	
	PKC	25			(7.4–9.5)	(2.2–2.9)	(3.4–5.3)	(6.3–7.4)	0.8

[1] EWR, raw egg-white; EWC, cooked egg-white; PKC, cooked pork kidney.

[2] Figures in parentheses denote the range of biotin.

[3] Average of 4 days' collections.

distribution of the total biotin output between feces and urine.

Biotin balances of rats on egg-white and biotin-rich rations

According to the hypothesis that avidin fixes biotin in an unabsorbable combination, it would be expected that increasing the amount of raw egg-white in the ration would increase the biotin in the feces. The averages are in general agreement with this hypothesis. The biotin content of the feces from lots fed raw egg-white averaged 139% of the ingested biotin while the urine contained only 19%. Corresponding figures for lots fed cooked egg-white were 75% and 28%, respectively. The distribution in the latter group resembles that found in the stock ration group.

At the high levels of biotin in the ration, its fixation by avidin is especially apparent. The average fecal biotin on the raw egg-white rations containing cooked kidney exceeded by 3- to 5-fold the corresponding average biotin elimination on rations containing egg-white without kidney (lots I and IV; II and V). Likewise, the fecal biotin output was 2 to 3 times as much on the raw egg-white ration with pork kidney as on the cooked egg-white and pork kidney rations (lots V and VI; XI and XII). These data support the view that the avidin of thoroughly cooked egg-white does not retain its biotin-binding property.

The response of adult stock rats (lot X) to egg-white and high levels of biotin (lots XI and XII) was very marked. Both the urinary and fecal biotin eliminations were increased. With raw egg-white in the ration, the fecal biotin was 10-fold that on the stock ration, while a 4-fold increase occurred on the ration containing cooked egg-white.

There is clear evidence that biotin from some non-dietary source was eliminated in the feces of rats on egg-white rations. It will be noted (table 1) that the ratio of total daily biotin output to intake on most of the egg-white rations exceeded 1, and that 60 to 95% of the output was present in the feces.

The biotin in the urines, in contrast, represented only 5 to 40% of the daily biotin intake. The probable explanation is a synthesis of biotin somewhere in the digestive tract. The bacteria native to the intestinal tract are known to synthesize biotin (Landy et al., '42; Thompson, '42; Burkholder and McVeigh, '42). Direct evidence has also been reported to show that biotin synthesis occurs in animals (McElroy and Jukes, '40; Wegner et al., '41; Nielsen et al., '42; Mitchell and Isbell, '42) and in human subjects (Gardner et al., '43, '45, '46; Oppel, '42; Parsons et al., '42).

An indication of the site of the biotin synthesis is given by the amount of biotin found in the contents of the stomach, small intestine, cecum and large intestine. From table 2, it is evident that the biotin concentration in the cecum and large intestine was strikingly greater than that in the stomach and small intestine. This increase was as much as 3- to 20-fold with respect to the rats on the egg-white rations. The cecum and large intestine of stock rats contained 2.0 μg and 2.3 μg per gm of dry matter, respectively, as compared with 1.0 and 0.9 μg in the stomach and small intestine. Based on the total contents (see footnote 1, table 2) one-third of the total biotin in the tract was found in the cecum and large intestine. Likewise with egg-white in the ration, from 88–98% of the total biotin was present in the contents of these 2 sections.

The presence of large amounts of biotin in the ceca suggests biotin synthesis at this point in the digestive tracts. Mitchell and Isbell ('42) have presented data which show that intestinal flora in the cecum may contribute largely to the rat's supply of several of the B-vitamins and indicate that these vitamins are absorbed by the body tissues.

Another possible source of non-dietary fecal biotin is the withdrawal and elimination of body stores of biotin. From the standpoint of the biotin concentration in the livers and kidneys (table 2) there is obviously no evidence of withdrawal of body stores due to high levels of raw egg-white. The excess of biotin output over intake for long periods of

TABLE 2

Biotin concentration in the contents of the digestive tract and in the organs and feces of albino rats on stock and egg-white rations.

LOT	BIOTIN IN RATION	BIOTIN IN THE CONTENTS OF THE DIGESTIVE TRACT AND IN THE ORGANS [1]						BIOTIN IN FECES PER DAY	
		Number of rats	Stomach	Small intestine	Cecum	Large intestine	Livers and kidneys	No. of rats	Biotin
	μg/gm		μg/gm	μg/gm	μg/gm	μg/gm	μg/gm		μg/gm
IV	1.2	3	2.2 (2.1–2.3)	6.2 (1.0–9.4)	20.0 (10.1–32.1)	41.1 (32.2–50.0)	1.1 (0.8–1.4)	3	33.8 (30.6–38.7)
V	1.1	2	2.6 (0.6–4.6)	4.9 (4.7–5.1)	14.0 (13.6–14.4)	15.7 (10.5–20.9)	2.7 (1.9–3.4)	2	16.6 (15.3–17.9)
VIa	1.1	3	1.3 (0.8–1.7)	1.5 (0.5–3.5)	5.6 (4.2–6.3)	7.1 (4.1–9.1)	1.4 (1.2–1.6)	2	9.3 (7.6–11.0)
VIIa	0.3	3	0.8 (0.2–1.3)	1.3 (1.0–1.4)	9.7 (8.4–11.8)	10.2 (6.8–13.5)	1.0 [2]	11	7.4 (3.6–12.6)
VIIIa	0.6	3	0.5 (0.3–0.7)	1.8 (0.3–3.0)	12.9 (10.7–14.0)	12.9 (11.4–14.4)	6.0 (1.3–16.3)	5	10.2 (8.0–15.4)
X and XIII [3]	0.2	9	1.00 (0.3–1.3)	0.9 (0.2–2.0)	2.0 (1.2–3.2)	2.3 (1.6–3.8)	0.9 (0.6–1.0)	4	1.1 (0.8–1.3)

[1] Average total weight of dry material as follows: contents of stomach 0.6 gm; small intestine 0.8 gm; cecum 0.5 gm; large intestine 0.4 gm; livers and kidneys lot IV 2 gm; V 2.7 gm; VI 2.8 gm; VIIa 2 gm; VIIIa 1.6 gm; XIII 4.0 gm.

[2] Liver and kidneys for 1 rat only.

[3] Lot X was composed of 4 animals, lot XIII of 5.

time is too great to assume that it could have originated from body stores.

The data in table 2 also give some indication of the comparative absorbability of biotin and the other constituents of the ration. As would be expected if the biotin-avidin complex is unavailable, an increase was found in the biotin concentration of the contents of the small intestine over the contents of the stomach and over that of the ration. This is particularly evident on raw egg-white rations containing cooked pork kidney (lots IV and V). The data indicate that part of the ingredients of the ration was absorbed from the small intestine faster than was the biotin; especially was this true where large amounts of biotin were present in a presumably unabsorbable form. Since synthesis of the B-vitamins is believed to occur in the digestive tract at some place beyond the small intestine, this increase in the small intestine is probably not accounted for by biotin synthesis.

SUMMARY

Evidence is presented that biotin from some non-dietary source was eliminated in the feces of rats on rations containing raw egg-white. The excess biotin in the urine and feces over that contained in the ration ingested ranged from 0.6 µg to 9.4 µg per day. The excess biotin presumably originated from synthesis of biotin by intestinal microorganisms rather than from the withdrawal of biotin stores in the liver, kidney or other body tissues. The main site of synthesis in the digestive tract appeared to be the cecum and large intestine.

Of the total biotin eliminated from 5 to 40% was found in the urine and from 60 to 95% was present in the feces.

The fixation of biotin by avidin was more apparent at the higher levels of biotin in the ration.

LITERATURE CITED

BURKHOLDER, P. R., AND I. MCVEIGH 1942 The synthesis of vitamins by intestinal bacteria. Nat. Acad. Sci., *28:* 285.

DU VIGNEAUD, V., K. DITTMER, K. HOFMANN AND D. B. MELVILLE 1942 Yeast-growth-promoting effect of diaminocarboxylic acid derived from biotin. Proc. Soc. Exp. Biol. Med., *50:* 374.

EAKIN, R. E., E. E. SNELL AND R. J. WILLIAMS 1940 A constituent of raw egg-white capable of inactivating biotin in vitro. J. Biol. Chem., *136:* 801.

——— 1941 The concentration and assay of avidin, the injury-producing protein in raw egg white. J. Biol. Chem., *140:* 535.

GARDNER, J., A. L. NEAL, W. H. PETERSON AND H. T. PARSONS 1943 Biotin, pantothenic acid, and riboflavin balances of young women on a milk diet. J. Am. Diet. Asso., *19:* 683.

GARDNER, J., H. T. PARSONS AND W. H. PETERSON 1945 Human biotin metabolism on various levels of biotin intake. Arch. Biochem., *8:* 339.

——— 1946 Human utilization of biotin from various diets. Am. J. Med. Sci., *211:* 198.

GYÖRGY, P., C. S. ROSE, R. E. EAKIN, E. E. SNELL AND R. J. WILLIAMS 1941 Egg white injury as the result of nonabsorption or inactivation of biotin. Science, *93:* 477.

LAMPEN, J. O., A. A. KLINE AND W. H. PETERSON 1941 The determination of biotin by a microbiological assay. Proc. Am. Soc. Biol. Chem., J. Biol. Chem., *140:* lxxiv.

LANDY, M. L., D. M. DICKINS, M. M. BICKING AND W. R. MITCHELL 1942 Use of avidin in studies on the biotin requirement of microorganisms. Proc. Soc. Exp. Biol. Med., *49:* 441.

McELROY, L. W., AND T. H. JUKES 1940 Formation of the anti egg-white-injury factor (biotin) in the rumen of the cow. Proc. Soc. Exp. Biol. Med., *45:* 296.

MITCHELL, H. K., AND E. R. ISBELL 1942 Intestinal bacterial synthesis as a source of B vitamins for the rat. Univ. of Texas Pub., no. 4237, p. 125.

NIELSEN, E., G. M. SHULL AND W. H. PETERSON 1942 Response of bacteria, yeast and rats to peroxide-treated biotin. Intestinal synthesis of biotin in the rat. J. Nutrition, *24:* 523.

OPPEL, T. W. 1942 Studies of biotin metabolism in man. Am. J. Med. Sci., *204:* 856.

OSBORNE, T. B., AND L. B. MENDEL 1919 The nutritive value of the wheat kernel and its milling products. J. Biol. Chem., *37:* 572.

PARSONS, H. T. 1931 The physiological effects of diets rich in egg white. J. Biol. Chem., *90:* 351.

PARSONS, H. T., J. G. LEASE AND E. KELLY 1937 The interrelationship between dietary egg white and the requirement for a protective factor in the cure of the nutritive disorder due to egg white. Biochem. J., *31:* 424.

PARSONS, H. T., J. GARDNER AND C. T. WALLIKER 1940 Possible significance of fecal concentrations of the factor protective against egg white injury. Proc. Am. Inst. of Nut., J. Nutrition, *19:* 19.

PARSONS, H. T., J. COLLARD, J. GARDNER, F. M. STRONG AND W. H. PETERSON 1942 Utilization by man of the vitamin B complex in fresh yeast. Fed. Am. Soc. Exp. Biol., Fed. Proc., *1:* 129.

SNELL, E. E., R. E. EAKIN AND R. J. WILLIAMS 1940 A quantitative test for biotin, its occurrence and properties. J. Am. Chem. Soc., *62:* 175.

THOMPSON, R. C. 1942 Synthesis of B vitamins by bacteria in pure culture. Univ. of Texas Pub., no. 4237, p. 87.

WEGNER, M. I., A. N. BOOTH, C. A. ELVEHJEM AND E. B. HART 1941 Rumen synthesis of the vitamin B complex on natural rations. Proc. Soc. Exp. Biol. Med., *47:* 547.

WOOLLEY, D. W., AND L. G. LONGSWORTH 1942 Isolation of an antibiotin factor from egg white. J. Biol. Chem., *142:* 285.

A STUDY OF ASCORBIC ACID METABOLISM OF ADOLESCENT CHILDREN[1]

CLARA A. STORVICK, MARGARET L. FINCKE, JEANNE PERKINS QUINN AND BESSIE L. DAVEY

School of Home Economics, Oregon State College, the Oregon Agricultural Experiment Station, Corvallis; and the Bureau of Human Nutrition and Home Economics, United States Department of Agriculture, Washington, D. C., Cooperating

(Received for publication November 14, 1946)

Studies of the human requirement for ascorbic acid have been made with adults and with children up to 12 years of age. As far as we have been able to ascertain, the requirements of adolescents have not been determined experimentally. This study was undertaken to provide some quantitative data for the estimation of the ascorbic acid need of children during early adolescence.

Children living at the Children's Farm Home, Corvallis, Oregon, served as subjects. These children, about 160 in number and ranging in age from 5 to 18 years, are housed in 8 cottages at the Children's Farm Home. Boys and girls live at separate cottages, except at the "health cottage," where a registered nurse resides, and which houses convalescents and children requiring special care. In order to simulate family life, children of different ages live in each cottage, and each cottage has its own cook and house-mother. All food is supplied through a central commissary, but its use depends upon the choices of the cooks.

[1] Published as Technical Paper 498 with the approval of the Director of the Oregon Agricultural Experiment Station and the Chief of the Bureau of Human Nutrition and Home Economics, U.S. Dept. of Agriculture. Contribution of the School of Home Economics of Oregon State College, the Oregon Home Economics Experiment Station and the Bureau of Human Nutrition and Home Economics, U. S. Dept. of Agriculture, Washington, D. C.

A survey of the plasma ascorbic acid values of 81 children between the ages of 11 and 18 was made. Eight of these children, aged 12 to 14 years, also served as subjects in an experimental study. Three levels of ascorbic acid intake were tested for 1 week each with all subjects. One of the main purposes of the experiment was to study the effect on the plasma ascorbic acid concentration of the daily allowance of ascorbic acid which has been recommended by the National Research Council ('45).

SURVEY OF PLASMA ASCORBIC ACID VALUES OF ADOLESCENTS

Throughout the entire study the determination of reduced ascorbic acid in plasma was made by the micro-method of Farmer and Abt ('36), using samples of blood obtained by fingerprick. During the survey, the blood samples were obtained either before breakfast or just before lunch if the breakfast had contained no fruit. Values for plasma ascorbic acid were obtained from 43 boys and 38 girls between the ages of 11 and 18. The results in terms of mean, average deviation, and range are summarized for each age in table 1.

TABLE 1

A summary of the plasma ascorbic acid determination on adolescents at the Children's Farm Home.

AGE	NUMBER OF CHILDREN	MEAN	AVERAGE DEVIATION	RANGE
		mg %	*mg %*	*mg %*
11	10	0.60	0.15	0.31–0.97
12	19	0.53	0.22	0.17–0.95
13	21	0.62	0.20	0.08–1.14
14	18	0.56	0.16	0.06–1.07
15	6	0.43	0.13	0.26–0.61
16	4	0.62	0.14	0.51–0.90
17	3	0.72	0.15	0.50–0.84

The Committee on Vitamins of the American Academy of Pediatrics, headed by Allan M. Butler ('40), reports that a plasma ascorbic acid value of 0.60 mg % may be regarded

as adequate. Seventy-one per cent (27) of the plasma levels of the girls and 42% (18) of those of the boys of the Children's Farm House fell below this level. The mean plasma value for the entire group tested, however, was 0.56 mg %, which very nearly meets this standard.

EXPERIMENTAL STUDY

The experimental study was designed to compare the daily values for ascorbic acid in the plasma of adolescents when on their usual diet, when saturated with ascorbic acid, and when receiving the daily allowance of ascorbic acid which has been recommended by the National Research Council ('45). A description of the subjects is included in table 2. The girls

TABLE 2

Description of experimental subjects.

SUBJECT	AGE		HEIGHT	MEAN WEIGHT DURING STUDY
	years	*months*	*inches*	*lbs*
Raymond	13	5	62.0	98
James	12	11	64.0	95
Jack	12	4	60.0	86
Jim	12	4	61.5	82
Jo	14	6	64.0	134
Stasia	13	9	65.5	101
Betty Lou	13	8	64.5	110
Sharon	12	3	62.0	107

lived at one cottage, and the boys lived at another. All of the children were apparently healthy, and examination of their medical records showed no evidence of abnormalities.

All of the foods which the subjects ate were weighed and recorded during a period of 3 weeks for each group. Samples of foods which might be presumed to contain ascorbic acid, were analyzed [2] after each meal by the method of Loeffler and Ponting ('42). Throughout the experimental study, blood samples were taken daily before breakfast.

[2] Details of the experimental methods and procedures, as well as complete tables of the results of food analyses, will be available as a technical bulletin published by the Oregon Agricultural Experiment Station.

Each experimental period of 3 weeks was divided into 3 parts. During the first week, the children ate the regular diet which was served in their cottage, the second week served as a saturation period, and during the third week, each child received the total amount of ascorbic acid recommended for his age and sex by the National Research Council ('45).

During the week on their regular diet, the first serving of each food was usually the same for each child, and if quantity permitted, it was a "standard" serving. During this week the children were allowed to have second servings of any food which they requested, as long as the supply lasted. Ascorbic acid intake varied, as did the intake of all other constituents during this week, according to the inclination of each child. In this way the effect of the diet, as usually served and eaten, on the ascorbic acid in the blood plasma, could be observed.

The second week served as a saturation period. In addition to the regular diet, from which significant sources of ascorbic acid were removed and replaced by foods of low ascorbic acid content, the children received a supplement of 200 mg of pure ascorbic acid [3] just before breakfast.

During the third week the ascorbic acid allowed from food was limited to 20 mg per day. Pure ascorbic acid was given before breakfast, as the saturation supplement had been, in order to bring the total daily intake to the amount recommended by the National Research Council ('45). The girls received a total of 80 mg of ascorbic acid daily: 20 mg from food and 60 mg as crystalline ascorbic acid dissolved in redistilled water. Using the age groupings of the National Research Council ('45), the boys fell into different groups. The fraternal twins, Jack and Jim, who had recently passed their twelfth birthday, received 55 mg of ascorbic acid plus 20 mg in food making their total daily intake 75 mg. The older boys, James (12 years and 11 months old) and Raymond (13 years, 5 months) received a supplement of 70 mg of the crystalline ascorbic acid or a total daily intake of 90 mg.

[3] Acknowledgment is made to Merck and Company, Inc., for a generous supply of crystalline ascorbic acid.

Although all foods were weighed, the children were allowed to eat, ad libitum, bread, butter, meat, and other foods which contained no ascorbic acid.

Between-meal eating presented some problems. The girls were accustomed to receiving some food after school so they were provided with extra food in the afternoon. The director of the boys' cottage, however, did not permit between-meal eating, so no after-school lunch was provided for them. The investigators felt that they had the complete cooperation of the subjects during the course of the experiment.

RESULTS OF EXPERIMENTAL STUDY

Table 3 presents the results of daily fasting plasma ascorbic acid determinations, and the corresponding daily intakes of each child during the study. Since no effort was made to change the diet of the children during the first period, the means for the entire period are reported, with the average deviations from the mean, as well as the range of values obtained during the period. The first 2 days were omitted from the calculation of means for the saturation periods and of the period when the recommended allowance of the National Research Council ('45) was given, to eliminate the effect of the previous level of intake. Average deviations from the means for the last 5 days of these 2 periods as well as the range of values are reported with the means for each subject.

The first period of observation with the boys showed them to be receiving a mean daily ascorbic acid intake of 61 mg, and maintaining blood plasma levels well within the range of adequacy, according to Butler ('40). Daily variations were rather marked throughout the study for most of the boys. James, however, varied little from day to day except during the saturation week. In spite of the daily variation, some general trends are apparent. There was a slight but definite increase in blood plasma ascorbic acid during the saturation period for each boy, without exception. The plasma levels of all of the boys dropped immediately with the lowered intake of ascorbic acid on the fifteenth day, and except for the daily

TABLE 3

Total daily intake and plasma ascorbic acid values for all subjects during 3 consecutive 1-week experimental periods.

SUBJECT	WEEK OF USUAL DIET MEAN ASCORBIC ACID		SATURATION WEEK ASCORBIC ACID			LAST 5 DAYS OF N.R.C. RECOMMENDED ALLOWANCE MEAN ASCORBIC ACID		
	Intake	Plasma	Mean intake	Mean plasma	Highest plasma value	Intake	Intake	Plasma
	mg/day	*mg %*	*mg/day*	*mg %*	*mg %*	*mg/day*	*mg/kg*	*mg %*
Raymond	59.1 ± 25.0 [1] (13–104) [2]	0.78 ± 0.10 (0.57–1.04)	233.8 ± 3.8 (227–243)	1.48 ± 0.19 (1.21–1.81)	1.37 [3]	90.0 ± 0.0	2.0	0.91 ± 0.05 (0.85–0.99)
James	62.1 ± 25.6 (13–114)	0.73 ± 0.03 (0.66–0.80)	236.2 ± 5.4 (227–243)	1 05 ± 0 08 (0.94–1.17)	1.17	90.0 ± 0.0	2.1	0.84 ± 0.03 (0.80–0.91)
Jack	60.3 ± 24.6 (13–104)	0.75 ± 0.09 (0.53–0.88)	236.2 ± 5.4 (227–243)	1.05 ± 0.03 (0.98–1.10)	1.10	75.0 ± 0.0	1.9	0.88 ± 0.07 (0.78–1.01)
Jim	62.0 ± 24.6 (13– 94)	0.75 ± 0.09 (0.49–0.97)	234.8 ± 5.0 (227–243)	1.19 ± 0.05 (1.07–1.25)	1.25	75.0 ± 0.0	2.0	0.98 ± 0.06 (0.86–1.03)
Jo	50.1 ± 15.9 (21– 88)	0.44 ± 0.06 (0.29–0.54)	215.4 ± 5.3 (207–223)	0.92 ± 0.15 (0.71–1.06)	1.06	80.0 ± 0.4 (79–81)	1.3	0.89 ±0.03 (0.84–0.96)
Stasia	41.6 ± 10.9 (24– 62)	0.47 ± 0.06 (0.28–0.59)	216.4 ± 4.5 (210–223)	0.96 ± 0 08 (0.84–1.13)	1.13	80.0 ± 0.4 (79–81)	1.7	0.86 ± 0.07 (0.74–0.94)
Betty Lou	42.9 ± 9.6 (29– 61)	0.23 ± 0.03 (0.18–0.29)	216.4 ± 4.5 (210–223)	1.14 ± 0.10 (0.89–1.35)	1.35	80.0 ± 0.4 (79–81)	1.6	1.21 ± 0.06 (1.13–1.35)
Sharon	47.0 ± 14.9 (25– 76)	0.20 ± 0.04 (0.13–0.31)	217.0 ± 5.2 (210–224)	0.97 ± 0.08 (0.76–1.03)	1.03	80.0 ± 0.4 (79–81)	1.6	0.91 ± 0.02 (0.86–0.95)

[1] Average deviation.

[2] Range.

[3] Raymond's value before illness.

variations, they remained at levels lower than their saturation levels, varying around 0.9 mg %.

During the third period when the subjects were in 2 age groupings, no difference was observed between the plasma levels for the 13-year-old boys receiving 90 mg per day and the 12-year-old boys receiving 75 mg per day.

The outstanding feature of this period is the peak reached by Raymond on the fourteenth day. He had been found to have a fever of 102½°F. after supper on the twelfth day. He was given 2 tablets of aspirin and 1 tablespoon of citro-carbonate by the house-mother, and he did not report for a blood sample the next morning. He remained in bed with a slight fever during that day, although he was kept as a subject. The next morning his plasma level had reached 1.81 mg %. Raymond had no fever during that day, and the following morning his plasma level was still much higher than that of the other children, namely, 1.54 mg %.

The possibility that the ingested aspirin or citro-carbonate might have caused the rise in Raymond's plasma value was considered. However, no increase in the concentration of ascorbic acid was noted in normal subjects at the end of 4, 24, 28 and 48 hours after the administration of aspirin and, later, in a similar study after the administration of citro-carbonate. The rise in Raymond's plasma, therefore, remains unexplained.

The 4 girls (table 3) started the study with a mean dietary intake of 44 mg of ascorbic acid per day. Their plasma levels seemed to be grouped into 2 pairs: Jo and Stasia showed plasma levels markedly above those of Betty Lou and Sharon. The variations in intake and in plasma ascorbic acid were small from day to day. After the beginning of the saturation period the increases in plasma ascorbic acid of all the girls were abrupt and of about the same degree. When apparent saturation levels had been attained, daily variations for all of the subjects were marked. This confirms the observations of Storvick and Hauck ('42), who reported wider variations in daily plasma values on high intakes than on low intakes

of ascorbic acid. Variations were especially marked for Jo, who claimed to have been ill [4] on the eleventh and twelfth days of the study.

Data for the first 2 days of the 80 mg period showed the same variation and heights as those during the saturation period, but for the last 5 days, variations were less marked. Sharon "leveled off" at about 0.9 mg %, which seems to be about 0.1 mg % lower than her saturation level. Stasia showed the greatest daily variation during the third period, with values ranging from 0.94 to 0.74 mg % during the last 5 days. Betty Lou dropped steadily from a near-peak of 1.35 to 1.13 mg %, a value lower than any since the beginning of her saturation values. Jo was the only one of the 4 girls whose data showed a definite upward climb after the first drop on the 80 mg level.

It will be observed that while the boys' intake for the first period is higher than the girls', there is an even greater difference in their plasma levels. This may be due in part to the difference in developmental age between the 2 groups, or the low values for the girls may reflect subsistence on a diet lower in ascorbic acid for a considerable time before the study began.

Peak values during saturation were about the same for both groups, but the boys attained their peaks somewhat before the girls, and their values dropped more abruptly when the intake was reduced.

While it would have been interesting to know whether the children, and especially Betty Lou, could maintain for prolonged periods these high plasma levels on the intake recommended by the National Research Council ('45), the results for this week suggest that their plasma values would be maintained above the level of adequacy according to Butler ('40).

DISCUSSION

In attempting to evaluate the results of this study, the findings of other research workers need to be reviewed. Investi-

[4] This subject was somewhat unstable. The nurse reported no evidence of illness.

gators have used various approaches to the problem of determining the ascorbic acid requirement of man. Some of the studies are based on plasma ascorbic acid levels, others on urinary excretion levels, and still others combine and compare the two. In order to study requirement, the daily intake of ascorbic acid must be known.

Some investigators have questioned the existence of any relationship between ascorbic acid requirement and body weight (Hathaway and Meyer, '41; Ralli et al., '39). Nevertheless, many workers have reported their results in milligrams per kilogram of body weight. Of 30 adults whose requirements for tissue saturation were studied by Belser, Hauck and Storvick ('39); Ralli, Friedman and Sherry ('39); Todhunter and Robbins ('40); Storvick and Hauck ('42); Fincke and Landquist ('42); and Kline and Eheart ('44), the estimated requirements of 24 fell between 1.3 and 1.8 mg per kg. Bryan et al. ('41) concluded on the basis of a survey that there is a close relationship between ascorbic acid intake per kg of body weight and fasting plasma ascorbic acid values. In their experiment, intakes of 1.7 to 1.9 mg per kg resulted in high fasting plasma ascorbic acid values, typical of tissue saturation. Fincke and Landquist ('42) found that ascorbic acid intakes of 0.8 to 1.2 mg per kg were necessary to maintain fasting plasma ascorbic acid values of about 0.8 mg % in 4 adult subjects, and Dodds and MacLeod ('44) observed that 1 mg per kg was adequate to cause a gradual increase in plasma values for 12 subjects who had been moderately depleted before being given increasing levels of ascorbic acid.

The 31 mg intake found by Hathaway and Meyer ('41) to maintain tissue saturation in 4 preschool children, was equivalent to 1.5 to 1.8 mg per kg for these children. The 5 children from 7 to 12 years of age studied by Roberts and Roberts ('42), required from 1.7 to 2.4 mg per kg to maintain tissue saturation. Thus, on the basis of body weight, requirements for tissue saturation of these children and of adults do not appear to differ greatly, although individual variations are apparent.

In the present study, mean fasting plasma values of from 0.9 to 1.2 mg % were maintained by the 4 adolescent girls on the allowance recommended by the National Research Council ('45), which was equivalent to 1.3 to 1.7 mg per kg. The National Research Council allowance, which was equivalent to 1.9 to 2.1 mg per kg for the 4 boys, maintained mean fasting plasma values from 0.8 to 1.0 mg % during the last 5 days of the 1-week period on this intake (see table 3). For these young adolescents, the allowances of ascorbic acid recommended by the National Research Council ('45) resulted in fasting plasma ascorbic acid values below those individually attained when saturation doses were given, although for 1 week these allowances were adequate to maintain plasma values which are associated with "good" stores of ascorbic acid. This first study on the ascorbic acid requirements of young adolescent boys and girls suggests that they may require somewhat more ascorbic acid per kilogram of body weight to maintain tissue saturation than do younger children or adults.

CONCLUSIONS

1. The daily allowance of ascorbic acid recommended for adolescents by the National Research Council ('45), as tested during this study, resulted for each subject in plasma values below those attained during the saturation period, but well above those considered adequate by Butler ('40). It would probably be advisable during the strain of adolescent growth to maintain all children near saturation, and since the recommended allowance accomplishes this in these subjects, diets may well be planned to include this amount for every child.

2. A mean plasma ascorbic acid concentration of 0.56 mg % was obtained from the survey of 81 children between 11 and 18 years of age residing at the Children's Farm Home. This value is about that (0.60 mg %) considered adequate by Butler.

LITERATURE CITED

BELSER, W. B., H. M. HAUCK AND C. A. STORVICK 1939 A study of the ascorbic acid intake required to maintain tissue saturation in normal adults. J. Nutrition, *17:* 513.

BRYAN, A. H., D. F. TURNER, R. L. HUENEMANN AND G. LOTWIN 1941 The relation between plasma and dietary ascorbic acid. Am. J. Med. Sci., *202:* 77.

BUTLER, A. M. 1940 Report of the Committee on Vitamins of the American Academy of Pediatrics, p. 14.

DODDS, M. L., AND F. L. MACLEOD 1944 Blood plasma ascorbic acid values resulting from normally encountered intakes of this vitamin and indicated human requirements. J. Nutrition, *27:* 77.

FARMER, C. J., AND A. F. ABT 1936 Determination of reduced ascorbic acid in small amounts of blood. Proc. Soc. Exp. Biol. Med., *34:* 146.

FINCKE, M. L., AND V. L. LANDQUIST 1942 The daily intake of ascorbic acid required to maintain adequate and optimal levels of this vitamin in blood plasma. J. Nutrition, *23:* 483.

HATHAWAY, M. L., AND F. L. MEYER 1941 Studies on the vitamin C metabolism of 4 preschool children. J. Nutrition, *21:* 503.

KLINE, A. B., AND M. S. EHEART 1944 Variation in the ascorbic acid requirements for saturation of 9 normal young women. J. Nutrition, *28:* 413.

LOEFFLER, A. J., AND J. D. PONTING 1942 Ascorbic acid. Rapid determination in fresh, frozen or dehydrated fruits and vegetables. Ind. Eng. Chem., Anal. Ed., *14:* 846.

NATIONAL RESEARCH COUNCIL 1945 Committee on Food and Nutrition. Recommended dietary allowances. Revised. Reprint and Circular Series, no. 122, p. 2.

RALLI, E. P., G. J. FRIEDMAN AND S. SHERRY 1939 The vitamin C requirement of man. Estimated after prolonged studies of the plasma concentration and daily excretion of vitamin C in 3 adults on controlled diets. J. Clin. Invest., *18:* 705.

ROBERTS, V. M., AND L. J. ROBERTS 1942 A study of the ascorbic acid requirements of children of early school age. J. Nutrition, *24:* 25.

STORVICK, C. A., AND H. M. HAUCK 1942 Effect of controlled ascorbic acid ingestion upon urinary excretion and plasma concentration in normal adults. J. Nutrition, *23:* 111.

TODHUNTER, E. N., AND R. C. ROBBINS 1940 Observations on the amount of ascorbic acid required to maintain tissue saturation in normal adults. J. Nutrition, *19:* 263.

A STUDY OF SEX DIFFERENCES IN THE COMPOSITION OF RATS, WITH EMPHASIS ON THE LIPID COMPONENT

SEX DIFFERENCE IN SUSCEPTIBILITY TO ESSENTIAL FATTY ACID DEFICIENCY WITH HIGH AND LOW FAT DIETS [1]

HAROLD G. LOEB [2] AND G. O. BURR [3]
Departments of Physiological Chemistry and Botany, University of Minnesota, Minneapolis

ONE FIGURE

(Received for publication January 6, 1947)

In the course of an investigation of the influence of essential fatty acids on various fat fractions of the albino rat, data were obtained on the composition of the experimental animals in terms of total lipid, non-lipid solids, and water content. These findings augment the limited data in the literature, particularly with regard to the comparative fat storage of male and female animals.

Boycott and Damant ('08) in a study of the fat content of male and female rats, guinea pigs, and mice stated that females tend to have a higher fat content. For rats, 41 males and 42 females raised on the ordinary laboratory diet, the detailed composition of which is not given, they obtained average values for the total fatty acids of 4.4 and 5.6%, respectively.

[1] Taken from a thesis presented to the Graduate Faculty of the University of Minnesota (1940) in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Rockefeller Foundation Fellow; present address, Kabat-Kaiser Institute, 2633 16th St., N.W., Washington 9, D.C.

[3] Present address, Experiment Station, Hawaiian Sugar Planters' Association, Honolulu 4, Hawaii, U.S.A.

Bachmann and coworkers ('38), reporting on the body constituents of albino rats on sugar diets, found no appreciable difference in fat content of males and females.

The data presented below constitute evidence for a sex difference in fat storage only when a specific dietary regimen is employed.

EXPERIMENTAL

At weaning time albino rats were given diet 551-B containing 84% sucrose, 12% crude casein, 4% salts,[4] and the necessary supplements which included 0.7 gm of dehydrated yeast [5] daily and adequate amounts of vitamin D,[6] carotene, and vitamin E (concentrate from wheat germ oil). This diet was continued for 4 weeks in order to deplete the fat reserves. During the following 8 weeks, which comprised the experimental period, the animals (litter mates) were placed on diets 560-B (60% sucrose, 20% lard, 15% casein, 5% salts [4]), 550-B (84% sucrose, 12% casein, 4% salts [4]), and diet 580-B which contained 71.7% hydrogenated coconut oil [7], 22.9% casein, and 6% salts.[4] In these diets purified casein was employed and constituted 12.5% of the total calories in each case. Six groups of rats were used, 3 male and 3 female, and each group contained 8 individuals. At the end of the experimental period the males of the control group (560-B) attained an average weight of 200 gm and the females 170 gm. Rats receiving the low-fat diet (550-B) attained weights of 170 and 150 gm for males and females, respectively. Those consuming the high-fat diet (580-B) attained weights of 108 and 104 gm for males and females, respectively. The growth curves are given in figure 1.

A second series of experiments was performed on rats prepared in the same way. For this purpose 6 males and 6 females were placed on diet 580-B, and 3 of each sex were maintained on diets 550-B and 560-B, respectively. Data were obtained

[4] Salt mixture 185 (McCollum and Simmonds, '18).

[5] Northwestern.

[6] Viosterol.

[7] Iodine value, 0.22. We are indebted to the research laboratories of Proctor and Gamble for this material.

on food and water consumption and fecal fat excretion, and the method for determining body fat was modified as described below. Since a different method of analysis was employed in this series, the data are reported separately in the tables, this phase of the investigation being designated as Series II and the one mentioned above as Series I.

At the end of the experimental period (the fifteenth week of life) the rats of Series I were killed by etherization after

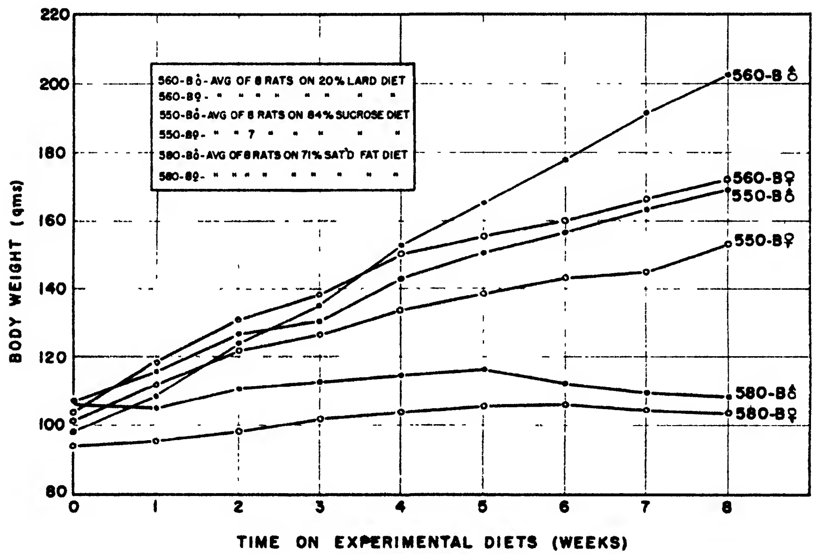


Fig. 1 Growth curves. Zero time represents the beginning of the eighth week of life. The first 3 weeks constitute the weaning period and the subsequent 4 weeks the period of fat depletion.

a 24-hour fast. The large intestine was discarded and the stomach and intestinal contents washed out with normal saline. The extraction followed, essentially, the procedure employed by Bloor ('26) in the extraction of beef heart muscle. The whole animal was comminuted in a meat chopper with fine cutter and dehydrated with 3 volumes of aldehyde-free alcohol (Dunlap, '06) for 1 to 2 hours. This aqueous alcohol solution was then decanted through a fluted filter paper and concen-

trated under carbon dioxide with reduced pressure to approximately 50 ml. It was then protected with carbon dioxide and set aside for petroleum ether extraction along with the main extract. The dehydrated tissue was extracted 3 times with 2 volume portions of boiling 95% alcohol under an atmosphere of carbon dioxide for 1, 1 and 0.5 hours, respectively. The combined extracts were concentrated under reduced pressure, with a stream of carbon dioxide bubbling through the solution, to a volume of 75 ml. This concentrate along with the one mentioned above was diluted with water and extracted 3 times with redistilled petroleum ether (b.p. 30–60°C.). The combined petroleum ether extracts were dried over anhydrous sodium sulfate overnight and filtered. The iodine number and total weight of the lipid were then determined. Weanling rats, 3 per analysis, were also analyzed in the same manner for comparison with the values obtained after maintenance on the diets described above.

After the extraction of lipid with hot alcohol the residual tissue was rinsed several times with small portions of anhydrous ether and the latter added to the alcohol solution. The residue was dried in air overnight and weighed. Although the weight remained constant, drying the residue at 105°C. overnight resulted in a decrease in weight approximating 7%. The oven-dried weight was employed to calculate the percentage of non-lipid solids. Water content was determined by difference.

In the second series of experiments the animals were treated as follows: After anesthetization the abdominal wall was opened and the blood withdrawn from the abdominal aorta for use in a study published elsewhere (Loeb, '42). The rats were then wrapped in paraffin paper and stored at —20°C. for approximately 2 months before analysis for carcass fat. The head, tail, and limbs were removed and the carcass digested for 4 hours in a 28% solution of potassium hydroxide made up with 50% alcohol. After cooling, the clear solution which contained a small quantity of insoluble material was carefully transferred through glass wool and made up to a known volume with 50% alcohol. The lipid content in terms of total

fatty acids plus unsaponifiable matter was determined in duplicate as follows: an aliquot portion was introduced into a large test tube and acidified with a hydrochloric acid solution of such strength as to leave the final alcohol concentration at 33%, by volume. After cooling, petroleum ether was added and the mixture well agitated. Centrifugation for 5 to 8 minutes gave a clean separation of layers and the petroleum ether layer was siphoned off. The extraction was repeated twice

TABLE 1

Iodine numbers of total lipids from whole animal (series I) and total fatty acids from carcass (series II).

GROUP	FAT IN DIET	SERIES I		SERIES II	
		No of rats	Mean [1] iodine no	No of rats	Mean [2] iodine no
Weanling rats		7 [3]	67.4 ± .35 [4]		
560-B ♂	20% lard	8	70.8 ± .62	2	76.9 ± .18
560-B ♀		8	68.9 ± .70	3	76.4 ± .18
550-B ♂	fat-free	7	61.6 ± .42	3	64.7 ± .30
550-B ♀		7	63.3 ± .45	3	65.0 ± .30
580-B ♂	71% hydrog.	8	45.5 ± 1.91	6	38.9 ± .41
580-B ♀	coconut oil	8	40.7 ± 1.27	6	39.0 ± .63

[1] Rosenmund-Kuhnhenn Method ('23).

[2] Wijs-Mercuric Acetate Method (Hoffman and Green, '39).

[3] This figure refers to the number of samples; 3 weanling rats per sample.

[4] Standard error of the mean calculated from the formula $\frac{S.D.}{\sqrt{N}}$ where S.D. represents the standard deviation, obtained from the expression $\sqrt{\frac{\Sigma x^2}{N}}$, in which x is the deviation from the mean.

more and after removing the solvent from the combined extracts the residue was dried in a vacuum oven at 60°C. for 1 hour, cooled in a desiccator and weighed. All of the duplicates checked well within 1%. For the determination of iodine numbers only the free fatty acids were used. Water was added to an aliquot portion of the original soap solution to reduce the alcohol concentration to 33%, by volume. The unsaponifiable fraction was extracted 3 times with petroleum ether and the remaining solution acidified and extracted as above.

Table 1 is a compilation of the mean iodine numbers of all the groups that were studied. Since different iodine number methods were employed in Series I and Series II, the 2 series are not strictly comparable to each other. However, the sex differences in unsaturation observed in Series I, if significant, are most likely due to the non-fatty acid portion of the total lipid. In Series II there is no suggestion of a sex difference in unsaturation of the total fatty acids of carcass.

Table 2 includes the mean values for the total composition of individuals studied in Series I. When analyzed statistically only group 580-B showed a significant sex difference in the storage of fat. The probability that this finding was due to chance was 1 in 59 ($P = 0.017$, Treloar, '42). There was no evidence of a sex difference in fat storage in groups 550-B and 560-B.

No difference in the percentage of non-lipid solids was observed between any of the groups regardless of sex or diet. Differences in water content due to sex could be shown statistically for groups 550-B and 580-B, those individuals with the higher fat content having also a lower water content. However, the probability was sufficiently close to the borderline for validity to warrant some reservation.

TABLE 2

Percentage composition of whole animals (series I).[1]

GROUP	DIET	SERIES I					
		No. of rats	Mean body weight	Water	Non-lipid solids	Total lipid	Total lipid
			gm	*%*	*%*	*%*	*gm*
Weanling rats		6[2]	127.4 ± 2.93	70.0 ± .81	24.7 ± 1.01	5.23 ± .02	6.66 ± .27
560-B ♂	20% lard	8	190.3 ± 6.31	67.0 ± .75	24.2 ± .47	8.85 ± .45	16.85 ± 1.24
560-B ♀		8	159.7 ± 2.55	65.5 ± .80	24.5 ± .42	10.01 ± .52	15.98 ± .92
550-B ♂	fat-free	8	154.5 ± 3.97	66.7 ± .55	25.0 ± .34	8.38 ± .59	12.95 ± 1.12
550-B ♀		7	131.9 ± 3.66	64.9 ± .38	25.8 ± .61	9.28 ± .56	12.24 ± .84
580-B ♂	71% hydrog.	8	99.2 ± 4.90	69.7 ± .68	24.9 ± .39	5.63 ± .69	5.58 ± .86
580-B ♀	coconut oil	8	95.2 ± 3.38	67.3 ± .74	24.7 ± .38	8.06 ± .54	7.67 ± .66

[1] For calculation of standard error of the mean see footnote in table 1.

[2] This figure refers to the number of samples; 3 weanling rats per sample.

Burr and Beber ('34) reported that rats on diet 550-B showed a higher metabolic rate, higher R.Q., and higher specific dynamic action of food than normal rats. In 1937, in a very carefully controlled series of experiments these same authors showed a marked difference in metabolic rate between rats maintained on diet 550-B and their controls which had been cured by administration of a curative oil. The difference between fat-deficient rats and animals from the stock colony receiving a sugar diet was still more striking. Since individuals

TABLE 3

Fat excretion and food and water consumption (series II).[1]

GROUP	NO OF RATS	DAILY FAT EXCRETION	DAILY FOOD CONSUMPTION		DAILY WATER INTAKE
		gm	*gm*	*cal*	*ml*
560-B ♂	3	0.035	7.19	34.5	8.7
560-B ♀	3	0.028	6.36	30.6	11.3
550-B ♂	3	0.014	6.76	26.0	11.1
550-B ♀	3	0.019	6.34	24.4	11.5
580-B ♂	6	0.028	3.83	28.0	8.5
580-B ♀	6	0.026	3.70	27.1	9.0

[1] These data were obtained on 1 or 2 groups of rats (3 per group), as indicated, during 6 weeks of the experimental period.

on diet 580-B consume as many calories as those on the fat-deficient diet (550-B)[8] (table 3) while at the same time undergoing scarcely any growth (fig. 1), and are losing no appreciable quantity of fat through the gut, it is reasonable to assume that their metabolic rate is even higher than that of rats on the fat-deficient diet. In addition, the greater fat content of females in group 580-B may be a reflection of a lower metabolic rate than that characterizing the males. It would appear from these observations that a deficiency of essential fatty acids has less effect on fat storage and metabolic rate in females than in males when both are maintained on high-fat diets.

[8] Burr and Burr ('30) showed that fat-deficient rats consume as much food as their cured controls.

DISCUSSION

The study of Deuel and coworkers ('44) of the comparative nutritive value of fats, using rats as experimental animals, has a bearing on the data reported above. They determined the composition of rats in terms of water, protein, lipid, carbohydrate, ash and calcium and concluded from their results that males have a higher water and protein content and a lower lipid, ash and calcium content. It is of interest to compare their results with the data we obtained on our control group, 560-B, which received a diet containing 20% lard (their animals consumed a diet containing approximately 30% fat). However, it should be noted that our rats were subsisting on a diet very low in fat for 4 weeks after weaning while theirs were placed on a 30% fat diet immediately after weaning. This probably accounts for the higher lipid values obtained by Deuel et al.

The values for protein, carbohydrate and ash obtained by Deuel and collaborators, when totalled, agree closely with our values for non-lipid solids, namely, 23.7% for males and 23.2% for females as against our values of 24.2% and 24.5% for males and females, respectively. We were not concerned with the carbohydrate and ash content. As for the total non-lipid solids, our difference between the means (0.3), was evidently not significant with a standard deviation ($\sqrt{\Sigma x^2/N}$) of 1.32 for the males and 1.20 for the females (8 rats in each group). In view of the large number of rats in the groups compared by Deuel et al. it would appear that the difference due to sex in protein as well as ash content is, in fact, a true statistical difference. It is well to note that these differences are obscured when total non-lipid solids are considered alone since the increased ash content found in females tends to mask their decreased protein content.

The sex difference in water content in our group 560-B (20% lard) was of the same magnitude as that observed by Deuel et al. but we are unable to establish statistical validity for this difference. The "t" value (Fisher, '38) was 1.3 whereas only a value in excess of 2.2 (the 5% level; i.e., 5

chances in 100 that the difference could be due to random sampling) could be accepted as being indicative of a valid difference. As mentioned above, the sex difference in water content for groups 550-B (fat-free diet) and 580-B (high saturated fat diet) was significant having "t" values of 2.3 and 2.2, respectively.

As for the lipid content our difference due to sex in group 560-B was of the same order of magnitude as that observed by Deuel et al. but, again, we could not establish statistical validity, the "t" value being 1.6. For group 550-B the "t" value was 1.1 while group 580-B showed a definite difference with a "t" value of 2.7.

Under the conditions of our experiment there was no statistically valid sex difference in the fat content of normal animals. The difference observed in our high-fat group (580-B) may therefore be ascribed to the lack of essential fatty acids which affects the males more than the females. It may well be, as Sinclair ('40) has suggested, that the requirement for essential fatty acids is greater on a high-fat diet. In this case one could infer that essential fatty acids are important in fat storage, and that females, probably through more efficient utilization of original stores of essential fatty acids, have a more effective fat-storing capacity than males. Or, to express it differently, the female may possess a more favorable fat economy when subjected to a condition of essential fatty acid deficiency while at the same time deriving most of its energy from fat. This is, essentially, an extension of an observation reported by Burr and Burr ('29) on rats consuming a fat-free diet (550-B). At the time of the growth plateau they found that males attain a weight equal to 70% of their controls receiving lard, while the weight of females is 80% of their controls. Furthermore, the eventual decline in weight proceeds more rapidly in males.

An examination of the iodine numbers in table 1 and the total lipid values in table 2 leads to the following conclusions: 20% lard in the diet contributes enough linoleic acid to maintain a normally high iodine number in the deposited fat; on

a fat-free, high carbohydrate diet much fat of low iodine number is synthesized and deposited; on a diet very rich in saturated fat and deficient in linoleic acid fat storage is less impaired in females than in males and there is a ready exchange of fatty acids in the tissues which results in very low iodine numbers. This supports the conclusion reached by metabolic studies that a severe deficiency of essential fatty acids does not prevent fat synthesis (Burr and Beber, '37) or mobilization of ingested fatty acids (Barnes, Rusoff and Burr, '42).

SUMMARY

1. Male and female albino rats were raised on 3 simplified diets; the control diet contained 20% lard while the other 2, deficient in essential fatty acids, were fat-free and rich in saturated fat (hydrogenated coconut oil), respectively. The composition of these individuals in terms of total lipid, total non-lipid solids, and water content was determined in addition to the unsaturation of body fat.

2. A sex difference in the unsaturation of fatty acids obtained from the body fat of rats raised on the 3 respective diets could not be established.

3. Evidence was presented showing that on a high-fat diet deficient in essential fatty acids females store more fat than males, and it was adduced that the latter are more sensitive to a deficiency in essential fatty acids than the females when both derive the bulk of their calories from fat.

4. Rats receiving a diet very high in saturated fat, but lacking essential fatty acids, are unable to effect an appreciable increase in the amount of total body lipid, but they readily exchange the dietary fatty acids with those in the tissues.

LITERATURE CITED

BACHMANN, G., J. HALDI, W. WYNN AND C. ENSOR 1938 The effect of a high glucose and a high fructose diet on the body weight and on the fat, glycogen, and nitrogen content of the liver and body of the albino rat. J. Nutrition, *16:* 229.

BARNES, R. H., I. I. RUSOFF AND G. O. BURR 1942 Influence of essential fatty acid deficiency on transport of fatty acids into the liver. Proc. Soc. Exp. Biol. Med., *51:* 235.

BLOOR, W. R. 1926 The distribution of unsaturated fatty acids in tissues. I. Beef heart muscle. J. Biol. Chem., *68:* 33.

BOYCOTT, A. E., AND G. C. C. DAMANT 1908 A note on the total fat of rats, guinea pigs, and mice. J. Physiol., *37:* 25.

BURR, G. O., AND A. J. BEBER 1934 Metabolism studies with rats suffering from fat deficiency. Proc. Soc. Exp. Biol. Med., *31:* 911.

——— 1937 Metabolism studies with rats suffering from fat deficiency. J. Nutrition, *14:* 553.

BURR, G. O., AND M. BURR 1929 A new deficiency disease produced by the rigid exclusion of fat from the diet. J. Biol. Chem., *82:* 345.

——— 1930 On the nature and role of the fatty acids essential in nutrition. J. Biol. Chem., *86:* 587.

DEUEL, H. J., JR., L. F. HALLMAN, E. MOVITT, F. H. MATTSON AND E. WU 1944 Studies of the comparative nutritive value of fats. J. Nutrition, *27:* 335.

DUNLAP, F. L. 1906 The preparation of aldehyde-free ethyl alcohol for use in oil and fat analysis. J. Am. Chem. Soc., *28:* 395.

FISHER, R. A. 1938 Statistical Methods for Research Workers, 7th Ed. Oliver and Boyd, London.

HOFFMAN, H. D., AND G. E. GREEN 1939 A rapid method for the determination of iodine number. Oil and Soap, *16:* 236.

LOEB, H. G. 1942 Effect of estradiol-benzoate on serum lipid of rats consuming a high fat diet. Proc. Soc. Exp. Biol. Med., *49:* 340.

MCCOLLUM, E. V., AND N. SIMMONDS 1918 A study of the dietary essential, water-soluble B, in relation to its solubility towards reagents. J. Biol. Chem., *33:* 63.

ROSENMUND, K. W., AND W. KUHNHENN 1923 Eine Neue Methode Zur Jodzahlbestimmung von Pyridinsulfatdibromid. Zeitschr. f. Untersuch. Nahr-u. Genussmittel, *46:* 154.

SINCLAIR, R. G. 1940 Growth of rats on high fat and low fat diets, deficient in the essential unsaturated fatty acids. J. Nutrition, *19:* 131.

TRELOAR, A. E. 1942 Random Sampling Distributions, Burgess Publishing Co., Minneapolis.

AMINO ACID CONTENT OF FEEDS

I. LEUCINE, VALINE, ISOLEUCINE AND PHENYLALANINE [1]

B. S. SCHWEIGERT

Nutrition Laboratory, Agricultural Experiment Station, and the School of Agriculture, Agricultural and Mechanical College of Texas, College Station

(Received for publication December 24, 1946)

The rapid development of microbiological methods for amino acid determinations has greatly facilitated their application to the estimation of amino acids in natural materials. Considerable data are now available to show that these techniques can be utilized to determine the amino acid composition of natural materials following hydrolysis without preliminary removal of fat, water or carbohydrate material. The importance of such data for the judicious selection of the protein components of the diet to assure adequacy of the essential amino acids is well recognized. The present study was initiated to obtain information on the amino acid content of the more common feeds, particularly those used in poultry feeding. The values for valine, isoleucine, phenylalanine and leucine are presented in this paper.

EXPERIMENTAL

The feed samples were obtained by random sampling of the supplies of commercial feeds available. For some of the feeds

[1] Acknowledgments are made to Frances Panzer and Patricia Sparks for technical assistance, to Mr. H. L. German and Dr. L. R. Richardson for supplying some of the feed samples, to Dr. C. M. Lyman and Dr. K. A. Kuiken for the analytically pure amino acid standards, to Lederle Laboratories for supplying the *L. casei* factor, to Merck and Co. for pyridoxamine and some of the amino acids and also to the Dow Chemical Co. for some of the amino acids used in this investigation.

(corn, corn gluten meal, meat and bone scraps, soybean oil meal, alfalfa leaf meal, and milo) additional samples were obtained from different sources. All samples were finely ground prior to assay to assure homogeneity.

The techniques used for the valine, leucine and isoleucine determinations were described previously (Schweigert et al., '44, '45). *L. arabinosus* was used as the test organism for these determinations. The determinations of phenylalanine were carried out by the use of *L. arabinosus* and *Leuconostoc mesenteroides* P-60. The medium used for the phenylalanine assay with *L. arabinosus* was the same as that used previously with the appropriate amino acid omitted from the basal medium. In the present studies *L. casei* factor and pyridoxamine were also included in the basal medium for *L. arabinosus* at levels of 0.02 and 1 µg per 10 ml of culture medium, respectively.

The medium used with *Leuconostoc mesenteroides* P-60 was the same as that used in earlier work (Riesen et al., '46) which is a modification of the medium described by Dunn and associates ('44). This organism was used for comparative tests, since the recent findings of Lyman ('46) and Lyman et al. ('46) show that *L. arabinosus* has the capacity to synthesize phenylalanine under certain test conditions. However, in the present work, excellent agreement was obtained with the use of both organisms for the determination of phenylalanine.

All amino acid standards used were critically tested for their purity to eliminate this factor as a possible source of error by comparing them with analytically pure standards. The amino acid values obtained are expressed on the basis of 100% activity for the l-isomer and no activity for the d-isomer.

The samples were hydrolyzed by autoclaving with 2N HCl at 15 lbs. pressure for 10 hours. This procedure has been found satisfactory for maximum liberation of amino acids (Schweigert et al., '44, '45; Riesen et al., '46). After hydrolysis, the samples were neutralized and aliquots taken for assay.

The results are expressed both as the per cent of each amino acid in the sample as analyzed and as the per cent in the crude

protein (calculated to 16% nitrogen). The determinations were made in duplicate or triplicate and all values were checked to within ± 5%. For comparison, results are also included for 3 purified proteins: casein, egg albumin and gelatin. The data are summarized in table 1.

To obtain further information on the usefulness of these individual values for calculating the amino acid content of rations composed of several sources of protein, 2 rations were analyzed for their amino acid content and the values compared with the calculated figures. These rations contained corn, oats, soybean oil meal, dried skim milk, corn gluten meal, fish meal, alfalfa leaf meal, meat and bone scraps and bone meal as constituents which supplied protein. The calculations were based on the analytical figures taken from the lots of feed used in preparation of the rations. The values found by calculation and determination are shown in table 2.

RESULTS AND DISCUSSION

The data presented in table 1 show that considerable variation exists in the amino acid content of feeds both when compared on the undried basis and on a protein basis. Large amounts of leucine were found in corn, corn gluten meal, milo and blood meal proteins. Isoleucine, however, is very low in blood meal. This is to be expected since several workers (Block and Bolling, '43; Brand and Grantham, '46; Devlin and Zittle, '44; and Orten et al., '45) have observed by analysis and in feeding tests that the principle protein, globin, is markedly deficient in this amino acid. The rice products tended to be low in all 4 amino acids.

The amino acid values obtained for different sources of the same feed were quite uniform for the corn products, milo, alfalfa leaf meal and soybean oil meal. Of the 4 amino acids tested, the values for phenylalanine were the most consistent, while those for leucine were the most variable. Rather divergent results were obtained for the various samples of meat and bone scraps. This is probably due to the varying composition of different lots of this feed. The amount of connective

TABLE 1

Amino acid content of feeds. All values are expressed as per cent of the undried sample and of the protein.

SAMPLE	PROTEIN (N × 6.25)	VALINE		ISOLEUCINE		PHENYLALANINE		LEUCINE	
		In the sample	In the protein	In the sample	In the protein	In the sample	In the protein	In the sample	In the protein
Corn	9.0	0.48	5.3	.38	4.2	0.40	4.4	1.04	11.6
Corn	9.06	0.47	5.2	.37	4.1	0.39	4.3	0.86	9.5
Corn	9.3	0.46	4.9	.38	4.1	0.39	4.2	0.89	9.6
Corn gluten meal	46.9	2.66	5.7	2.40	5.1	2.53	5.4	7.5	16.0
Corn gluten meal	49.0	2.78	5.7	2.43	5.0	2.57	5.2	7.6	15.5
Milo	11.6	0.57	4.9	0.51	4.4	0.47	4.1	1.38	11.9
Milo	12.4	0.55	4.4	0.51	4.1	0.48	3.9	1.14	9.2
Oats	11.6	0.66	5.7	0.52	4.5	0.52	4.5	0.74	6.4
Wheat	15.2	0.73	4.8	0.58	3.8	0.67	4.4	0.85	5.6
Wheat bran	16.0	0.82	5.1	0.54	3.4	0.53	3.3	0.83	5.2
Rice bran	16.0	0.70	4.4	0.50	3.1	0.47	2.9	0.69	4.3
Rice polish	15.6	0.59	3.8	0.40	2.6	0.40	2.6	0.57	3.7
Alfalfa leaf meal	18.6	1.10	5.9	0.96	5.2	0.79	4.2	1.28	6.9
Alfalfa leaf meal	18.5	1.04	5.6	0.93	5.0	0.75	4.1	1.17	6.3
Soybean oil meal	43.8	2.23	5.1	2.32	5.3	1.93	4.4	3.04	7.0
Soybean oil meal	44.8	2.32	5.2	2.14	4.8	2.04	4.5	3.16	7.1
Cottonseed meal	40.9	2.13	5.2	1.54	3.8	1.75	4.3	2.18	5.3
Linseed meal	42.7	2.15	5.0	2.2	5.2	1.83	4.3	2.13	5.0
Peanut meal	41.8	1.87	4.5	1.74	4.2	2.06	4.9	2.13	5.1
Cow peas, defatted	24.2	1.30	5.4	1.26	5.2	1.20	5.0	1.66	6.9
Fish meal	69.6	4.45	6.4	3.63	5.2	2.8	4.0	5.4	7.8
Meat and bone scraps	52.1	2.84	5.5	1.92	3.7	2.18	4.2	3.16	6.1
Meat and bone scraps	52.0	2.31	4.4	1.54	3.0	1.81	3.5	3.24	6.2
Meat and bone scraps	45.0	1.91	4.2	1.39	3.1	1.44	3.2	2.32	5.1
Blood meal	85.0	6.7	7.9	1.02	1.2	5.0	5.9	9.8	11.5
Sardine meal	65.1	4.08	6.3	3.27	5.0	2.57	3.9	4.69	7.2
Bone meal	33.1	1.06	3.2	0.65	1.96	0.76	2.3	1.29	3.9
Dried skim milk	35.4	2.8	7.9	1.97	5.6	1.58	4.5	3.42	9.7
Dried whey	10.6	0.68	6.4	0.66	6.2	0.35	3.3	0.99	9.3
Casein	95.7	6.7	7.0	5.9	6.2	5.2	5.4	9.35	9.8
Egg albumin	88.9	6.28	7.1	5.4	6.1	4.7	5.3	7.2	8.1
Gelatin	82.2	2.8	3.4	1.78	2.17	2.07	2.52	3.3	4.0

tissue, etc., in relation to the amount of muscle tissue would undoubtedly affect the amounts of the amino acids in the total protein. Furthermore, gelatin, derived from collagen and other connective tissue proteins, is rather low in these amino acids.

Some comparative values are available from the literature on the amino acid content of these feeds and are summarized by Block and Bolling ('45). The greatest discrepancies in the values are for valine and leucine. In general, the former were higher and the latter lower than those quoted by Block and Bolling. Results obtained microbiologically for some of these materials are published elsewhere (Kuiken et al., '43; Brand et al., '45; Stokes et al., '45; Hier et al., '45; McMahan and Snell, '44).

TABLE 2

Amino acid content of rations determined by actual analysis and calculated from the values obtained for the components.[1] *All values are expressed as per cent in the ration.*

RATION	LEUCINE	VALINE	ISOLEUCINE	PHENYLALANINE
No. 1 (determined)	2.49	1.17	1.06	1.07
No. 1 (calculated)	2.54	1.33	1.10	1.14
No. 2 (determined)	2.47	1.27	1.10	1.14
No. 2 (calculated)	2.64	1.42	1.21	1.19

[1] The amount of each amino acid contributed by the various protein ingredients was calculated on the basis of the percentage of that ingredient in the ration and from the data in table 1.

It is rather unlikely that a deficiency in leucine, valine, isoleucine or phenylalanine would be encountered with rations containing sufficient protein from a combination of feeds such as corn, oats, corn gluten meal, soybean oil meal, dried skim milk, fish meal, alfalfa leaf meal and meat and bone scraps. The leucine, valine, isoleucine and phenylalanine contents of poultry rations made up of these feeds were determined (table 2). The content is well above the estimated requirements for chickens, namely, 0.5% isoleucine, 1.5% leucine, 0.7% valine and phenylalanine, 0.5 to 1.5%, depending on the

level of tyrosine (Almquist and Grau, '44; Grau and Peterson, '46). No data, however, are available on the quantitative requirements of turkeys for these amino acids. The values obtained by calculating the amount of each amino acid furnished by the 6–8 protein components of the ration agree rather well with the values obtained when the entire ration was analyzed. The values for valine showed the greatest difference, although none of the determined and calculated values differed by more than ± 7%. Calculations based on these figures may not agree as well as observed in the present study when other sources of these feeds are incorporated in rations and such rations analyzed for their amino acid content.

When results become available on the quantitative requirements of all essential amino acids for various animals and on the amounts present in various feeds one may then interpret the adequacy of the diet in terms of the content of each amino acid. The important problem associated with availability of each amino acid can then be more easily investigated.

SUMMARY

1. The leucine, valine, isoleucine and phenylalanine contents of various feed materials have been determined by the use of microbiological methods.

2. Values for these amino acids in different lots of corn, corn gluten meal, alfalfa leaf meal, milo and soybean oil meal were quite uniform. More variability was observed between different samples of meat and bone scraps.

3. The differences between the amino acid content of 2 rations calculated from data on individual ingredients and determined by actual analysis were within ± 7%.

LITERATURE CITED

ALMQUIST, H. J., AND C. R. GRAU 1944 The amino acid requirements of the chick. J. Nutrition, *28:* 325.

BLOCK, R. J., AND D. BOLLING 1943 The amino acid yield from various animal and plant proteins after hydrolysis of the fat free tissue. Arch. Biochem., *3:* 217.

——— 1945 The Amino Acid Content of Proteins and Foods. Charles C. Thomas Publishing Co., Springfield.

BRAND, E., AND J. GRANTHAM 1946 Methionine and isoleucine content of mammalian hemoglobins. Its significance for nutritional and metabolic studies. J. Am. Chem. Soc., *68:* 724.

BRAND, E., F. J. RYAN AND E. M. DISKANT 1945 Leucine content of proteins and foodstuffs. J. Am Chem. Soc., *67:* 1532.

DEVLIN, H. B., AND C. A. ZITTLE 1944 A nutritional study of human globin in rats. J. Biol. Chem., *160:* 435.

DUNN, M. S., S. SHANKMAN, M. N. CAMIEN, W. FRANKL AND L. B. ROCKLAND 1944 The amino acid requirements of Leuconostoc mesenteroides P-60. J. Biol. Chem., *156:* 703.

GRAU, C. R., AND D. W. PETERSON 1946 The isoleucine, leucine and valine requirements of the chick. J. Nutrition, *32:* 181.

HIER, S. W., C. E. GRAHAM, R. FRIEDES AND D. KLEIN 1945 The microbiological determination of amino acids in animal proteins. J. Biol. Chem., *161:* 705.

KUIKEN, K. A., W. H. NORMAN, C. M. LYMAN, F. HALE AND L. BLOTTER 1943 The microbiological determination of amino acids I. Valine, leucine and isoleucine. J. Biol. Chem., *151:* 615.

LYMAN, C. M. 1946 Some chemical factors which influence the amino acid requirements of lactic acid bacteria. Abstracts of papers. Am. Chem. Soc., 110th meeting, p. 22B.

LYMAN, C. M., O. MOSELEY, S. WOOD, B. BUTLER AND F. HALE 1946 On the function of pyridoxine in lactic acid bacteria. J. Biol. Chem., *162:* 173.

MCMAHAN, J. R., AND E. E. SNELL 1944 The microbiological determination of amino acids I. Valine and arginine. J. Biol. Chem., *152:* 83.

ORTEN, J. M., J. E. BOURQUE AND A. U. ORTEN 1945 The inability of human or beef globin to support normal hematopoiesis in the rat without added isoleucine. J. Biol. Chem., *160:* 435.

RIESEN, W. H., B. S. SCHWEIGERT AND C. A. ELVEHJEM 1946 Microbiological determination of methionine in proteins and foodstuffs. J. Biol. Chem., *165:* 347.

SCHWEIGERT, B. S., J. M. MCINTIRE, C. A. ELVEHJEM AND F. M. STRONG 1944 The direct determination of valine and leucine in fresh animal tissues. J. Biol. Chem., *155;* 183.

SCHWEIGERT, B. S., I. E. TATMAN AND C. A. ELVEHJEM 1945 The leucine, valine and isoleucine content of meats. Arch. Biochem., *6:* 177.

STOKES, J. L., M. GUNNESS, I. M. DWYER AND M. C. CASWELL 1945 A uniform assay for the 10 essential amino acids. J. Biol. Chem., *160:* 35.

THE ROLE OF B_6-DEFICIENCY IN THE TRYPTOPHANE-NIACIN RELATIONSHIPS IN RATS [1]

FRED ROSEN,[2] J. W. HUFF[3] AND W. A. PERLZWEIG
Department of Biochemistry, Duke University School of Medicine, Durham, N. C.

(Received for publication December 31, 1946)

In several species of animals, the course of tryptophane metabolism is affected by the vitamin B_6 content of the diet. When the pyridoxine intake is adequate kynurenic acid is the metabolite which appears in the urine of rabbits, dogs and rats (Berg, '34; Correll et al., '38; Homer, '15). In rats and dogs maintained on a pyridoxine-deficient diet the normal metabolic derivative is replaced by xanthurenic acid (Lepkovsky and Nielsen, '42). Kynurenin has been postulated as the single intermediate through which the 2 end-products of tryptophane metabolism are derived (Lepkovsky et al., '43).

Data in a previous note (Rosen et al., '46) from this laboratory showed that a marked rise in the excretion of nicotinic acid and of N^1-methylnicotinamide occurred when *dl*- or *l*-tryptophane was administered orally or subcutaneously to rats. Since the normal metabolism of tryptophane in rats yields N^1-methylnicotinamide in addition to kynurenic acid, it was thought desirable to study the effect of a pyridoxine-free diet on this relationship.

[1] The authors wish to thank Miss Nancy Anne Hunter and Miss Betty Jean Peck for helpful technical assistance.

[2] Nutrition Foundation Fellow.

[3] Nutrition Foundation Fellow. Present address: Medical Research Division, Sharp and Dohme, Inc., Glenolden, Pa.

EXPERIMENTAL AND METHODS

Pyridoxine deficiency was induced in rats by feeding a ration of the following percentage composition: Vitamin-free casein,[4] 35; sucrose, 58; salts (Hubbell et al., '37), 4; cottonseed oil, 3. The ration was supplemented with 0.5 mg of thiamine, 1.0 mg of riboflavin, 2.5 mg of calcium pantothenate, 10 mg of inositol, 100 μg of vitamin K [5] and 100 mg of choline chloride per 100 gm. Each animal received 2 drops of cod liver oil twice weekly. A high protein (35% casein) diet was adopted because of the claim by Cerecedo and Foy ('44) that pyridoxine deficiency can be accelerated in rats by such diets.

A simple quantitative procedure for xanthurenic acid based on the formation of a green ferric salt in an alkaline medium (Lepkovsky et al., '43) was used for estimating this compound. To a volume (1–3 ml) of undiluted rats' urine in a graduated cylinder, adjusted to pH 8.1 with 1 ml of a saturated $NaHCO_3$ solution, 1 drop of 1.7% ferric ammonium sulfate is added. The contents are diluted to 10 ml with distilled water mixed by inversion, and poured into a calibrated Evelyn tube. A blank is prepared by diluting 1 ml of saturated $NaHCO_3$ and 1 drop ferric ammonium sulfate to 10 ml with distilled water. Readings were made in the Evelyn colorimeter using filter 620, set at 100 with the blank tube. Pure xanthurenic acid,[6] was used as a standard.

The N^1-methylnicotinamide was determined in a filtered sample of urine by the acetone-fluorometric method of Huff and Perlzweig ('47).

Six male rats (86–155 gm) maintained on the above diet for 30 days showed but slight acrodynia and complete cessation of growth. On the other hand the metabolic aspect of B_6 deficiency was definitely manifested by the excretion of appreciable amounts of xanthurenic acid in the urine after repeated doses of *dl*-tryptophane as shown in table 1. It can be seen from table 1 that as the pyridoxine deficiency became more

[4] Smaco.

[5] Synkavite.

[6] Kindly furnished by Sharp and Dohme, Inc.

TABLE 1

Twenty-four-hour excretion of N¹-methylnicotinamide and xanthurenic acid on pyridoxine-deficient diet after oral doses of tryptophane.

DAY	RAT 1		RAT 2		RAT 3		RAT 4		RAT 5		RAT 6		N'-ME. AVERAGE
	N'-Me [1]	Xant. [1]	N'-Me.	Xant.	N'-Me.	Xant.	N'-Me.	Xant.	N'-Me.	Xant.	N'-Me.	Xant.	
	μg	mg	μg	mg	μg	mg	μg	mg	μg	mg	μg	mg	μg
	After 11 days on pyridoxine-deficient diet												
12	40		19		61		146		80		75		70
	After 100 mg d,l-tryptophane orally												
13	200	5.3	590	1.8	340	4.0	841	3.0	750	1.4	652	9.2	562
14	193	1.6	187	0.4	85	1.7	267	0.8	250	0.3	260	2.3	207
15	113	0	148	0	90	0	104	0	256	0	125	0	139
	After 100 mg d,l-tryptophane orally												
16	298	6.0	620	4.1	254	14.1	682	6.5	718	3.7	435	6.2	501
17	183	1.0	200	0.4	106	1.4	177	0.7	237	0.6	187	1.2	180
18	56	0	102	0	85	0	106	0	194	0	136	0	103
21	55		70		33		67		119		60		67
	After 100 mg d,l-tryptophane orally												
22	83	7.6	276	10.8	83	15.9	220	14.5	375	9.8	151	9.1	175
23	77	0.6	93	0.6	35	3.6	57	2.2	125	1.0	60	0.9	74
24	50		42		22		57		119		52		57
	After 100 mg d,l-tryptophane orally												
26	79	7.2	240	9.4	40	13.1	127	7.1	150	17.5	120	9.9	126
27	39	0.6	52	0.6	13	2.3	42	1.4	116	1.3	43	0.4	51
	After 5 mg pyridoxine HCl daily for 4 days and then 100 mg d,l-tryptophane orally												
36 [2]	175	0	203	0	200	0	212	0	350	0	210	0	225
37	125		184		78		55		85		68		99

[1] *N'-Me.* denotes N¹-methylnicotinamide. *Xant.* denotes xanthurenic acid.

[2] On the twenty-ninth day 50 mg of N¹-methylnicotinamide was given orally to each rat (see text, p. 564).

acute, as measured by the increase in xanthurenic acid, there was a progressive decrease in the excretion of N^1-methylnicotinamide. Two control rats maintained for 35 days on the same diet but including 2 mg % pyridoxine excreted 3420 and 1690 μg N^1-methylnicotinamide in 48 hours after a dose of 100 mg *dl*-tryptophane. This is in contrast to the average value 177 μg found in the experimental rats after 26 days of pyridoxine depletion.

To check the possibility that the destruction of N^1-methylnicotinamide was not increased during the pyridoxine deficiency, each animal received 50 mg of N^1-methylnicotinamide by stomach tube. Approximately 16 to 28% was recovered in the urine in 48 hours, which indicates a normal rate of destruction of N^1-methylnicotinamide (Huff and Perlzweig, '46). In another series of pyridoxine-deficient rats, an oral dose of 25 mg of nicotinamide resulted in normal excretion of N^1-methylnicotinamide, indicating that the mechanism for methylating nicotinamide is not impaired in a vitamin B_6 deficient rat.

After 31 days on the pyridoxine-free diet, each rat received 5 mg of pyridoxine hydrochloride daily for 4 days before administration of 100 mg *dl*-tryptophane. The excretion of xanthurenic acid ceases abruptly after an oral dose of vitamin B_6, as was previously shown by Lepkovsky et al. ('43). But the disappearance of xanthurenic acid was accompanied by a relatively small rise in N^1-methylnicotinamide excretion after a dose of tryptophane, whereas in animals maintained on a complete diet, as the 2 control rats mentioned above, the usual response to a 100 mg dose of *dl*-tryptophane is the excretion of 1–3.5 mg per 48 hours.

For a period of 2 weeks after the initial 5 mg pyridoxine supplement each animal received 50 μg of B_6 daily. This resulted in an average weight increment of 24 gm per rat per week, indicating the overall remission of the pyridoxine deficiency. However, the low level of N^1-methylnicotinamide excretion after the test dose of tryptophane remained unaffected. During this period of B_6 supplementation the addi-

tion of biotin and folic acid or of brewers' yeast and Wilson liver (Fraction L) did not affect the results.

While the data given in this paper show the results on 6 rats only, for a period of 37 days, data involving experiments on 12 other rats, employing somewhat different diets and shorter periods of observation, yielded substantially the same results, namely, a significant decrease in N^1-methylnicotinamide excretion after a test dose of tryptophane. We have not found, to date, a single rat failing to exhibit this effect.

DISCUSSION

The data presented in this paper indicate that prolonged pyridoxine deficiency in half grown animals affects adversely the transformation of tryptophane to nicotinic acid in the rat. This metabolic defect is not rapidly restored after the animals ingest adequate amounts of the vitamin. That the hitherto observed abnormality in tryptophane metabolism involving the formation of xanthurenic acid is not directly related to the decrease of niacin formation is demonstrated by the simultaneous disappearance of xanthurenic acid from the urine.

The prolonged effect of B_6 deficiency on the synthesis of niacin and the failure to restore normal function even after 2 weeks of furnishing the vitamin point conceivably to changes in bacterial flora in the gut which are slow in developing. But the unlikelihood of the significant participation of the intestinal bacteria in the formation of niacin is emphasized by the extremely rapid response of rats to parenteral injections of tryptophane (Rosen et al., '46).

Furthermore, we attempted to test the relation of the

$$\begin{tikzcd} \text{tryptophane} \arrow[r] & \text{kynurenine} \arrow[r] \arrow[d, "\text{in } B_6 \text{ deficiency}"] & \text{kynurenic acid} \\ & \text{xanthurenic acid} & \end{tikzcd}$$

metabolic reaction (Lepkovsky et al., '43) to our problem. The administration of kynurenine, kynurenic acid and xanthurenic acid to rats on a complete diet failed to pro-

duce increased amounts of N^1-methylnicotinamide in the urine. This leads us to believe that the metabolic course from tryptophane to niacin in the rat is not via the above kynurenine pathway.

It was recently demonstrated that it is pyridoxal phosphate, rather than pyridoxine itself, which is involved in the enzymic systems concerned in transamination and decarboxylation of amino acids (Umbreit and Gunsalus, '45; Lichstein et al., '45) and in the synthesis of tryptophane (Umbreit et al., '46). Therefore, we thought it desirable to test the effect of administering pyridoxal upon the transformation of tryptophane to niacin in B_6-deficient rats. Subcutaneous and oral administration of 5 mg doses of pyridoxal daily proved as ineffective as pyridoxine itself in the experiments described above.

On the basis of our observations it is not possible at this time to propose a plausible hypothesis as to the possible mode of action of pyridoxine upon the tryptophane $\rightarrow$ niacin reaction. Until the enzyme systems involved in this transformation are more clearly defined this problem will probably remain unsolved.

SUMMARY

The omission of pyridoxine from the diet of rats results in a progressive decrease in the urinary excretion of N^1-methylnicotinamide after doses of tryptophane. This defect is only slightly overcome by the restoration of pyridoxine to the diet. The data indicate that the abnormal formation of xanthurenic acid in B_6 deficiency is not responsible for the impaired tryptophane to niacin transformation.

ACKNOWLEDGMENTS

Grants in aid of this study by the U. S. Public Health Service, The John and Mary Markle Foundation, and the Duke University Research Council are gratefully acknowledged.

The authors also wish to express their gratitude to Dr. S. Lepkovsky of the University of California for samples of kynurenine and xanthurenic acid; to Merck and Company,

Inc., Rahway, N. J., for the supply of pyridoxal and N[1]-methylnicotinamide; and to Sterling-Winthrop Research Institute, Rensselaer, N. Y., for the supply of *dl*-tryptophane.

LITERATURE CITED

BERG, C. P. 1934 The influence of optical activity on the utilization of tryptophane for growth and for kynurenic acid production. J. Biol. Chem., *104:* 373.

CERECEDO, L. R., AND J. R. FOY 1944 Protein intake and pyridoxine deficiency in the rat. Arch. Biochem., *5:* 207.

CORRELL, J. T., C. P. BERG AND D. W. COWAN 1938 The excretion of kynurenic acid in the bile and urine of the dog after the administration of kynurenic acid and *l*- and *dl*-tryptophane. J. Biol. Chem., *123:* 151.

HOMER, A. 1915 The relation between the administration of tryptophane to dogs and the elimination of kynurenic acid in their urine. J. Biol. Chem., *22:* 391.

HUBBELL, R. G., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, *14:* 273.

HUFF, J. W., AND W. A. PERLZWEIG 1946 The effect of CCl_4 poisoning on the fate of N[1]-methylnicotinamide in the rat. Fed. Proc., *5:* 149.

——————— 1947 The fluorescent condensation product of N[1]-methylnicotinamide with acetone. II. A sensitive method for the determination of N[1]-methylnicotinamide in urine. J. Biol. Chem., (In press).

LEPKOVSKY, S., AND E. NIELSEN 1942 A green pigment-producing compound in urine of pyridoxine-deficient rats. J. Biol. Chem., *144:* 135.

LEPKOVSKY, S., E. ROBOZ AND A. J. HAAGEN-SMIT 1943 Xanthurenic acid and its role in the tryptophane metabolism of pyridoxine-deficient rats. J. Biol. Chem., *149:* 195.

LICHSTEIN, H. C., I. C. GUNSALUS AND W. W. UMBREIT 1945 Function of the vitamin B_6 group: pyridoxal phosphate (codecarboxylase) in transamination. J. Biol. Chem., *161:* 311.

ROSEN, F., J. W. HUFF AND W. A. PERLZWEIG 1946 The effect of tryptophane on the synthesis of nicotinic acid in the rat. J. Biol. Chem., *163:* 343.

UMBREIT, W. W., AND I. C. GUNSALUS 1945 The function of pyridoxine derivatives arginine and glutamic acid decarboxylases. J. Biol. Chem., *159:* 333.

UMBREIT, W. W., W. A. WOOD AND I. C. GUNSALUS 1946 The activity of pyridoxal phosphate in tryptophane formation by cell-free enzyme preparation. J. Biol. Chem., *165:* 731.

THE EFFECT OF FAT LEVEL OF THE DIET ON GENERAL NUTRITION

I. GROWTH, REPRODUCTION AND PHYSICAL CAPACITY OF RATS RECEIVING DIETS CONTAINING VARIOUS LEVELS OF COTTONSEED OIL OR MARGARINE FAT AD LIBITUM [1]

HARRY J. DEUEL, JR., EMILY R. MESERVE, EVELYN STRAUB, CORNELIA HENDRICK AND BRADLEY T. SCHEER

Department of Biochemistry and Nutrition, University of Southern California School of Medicine, Los Angeles

TWO FIGURES

(Received for publication November 27, 1946)

The small amount of information now available indicates that there may be an optimum fat level in the diet. The requirement of experimental animals for minimal amounts of certain unsaturated fatty acids is now well established (Burr, '42) although the single human experiment of Brown et al. ('38) was not conclusive. The suggestion that certain eczematous conditions in infants may result from fat deficiency has received some support (Hansen, '33; Finnerud et al., '41).

On the other hand, there is some evidence that excessive amounts of fat in the diet may be harmful. Higgins ('30) has reviewed the earlier medical literature, and reports reduced ability to work on high-fat diets. Loewy and coworkers ('42)

[1] This work was carried out under a research grant from The Best Foods, Inc. The authors wish to acknowledge the helpful advice of Professor Anton J. Carlson of the University of Chicago, of Professor Arthur W. Thomas of Columbia University and of Dr. H. W. Vahlteich of The Best Foods, Inc., during the course of the experiments.

We are indebted to Merck and Co., Rahway, N. J., for the synthetic B vitamins; to the Winthrop Chemical Co., Albany, N. Y., for the crystalline vitamin D_2 and to Lederle Laboratories, Inc., for the folic acid.

observed increased erythrocyte destruction when large amounts of fat were added to the diet of dogs. Sako ('42) found a lowered resistance to pneumococci in mice which had received a high-fat diet (50%) for 6 weeks as compared with animals fed diets with less fat (23 or 5%).

Hoagland and Snider ('40) found greater gains in weight on diets containing 30 to 55% of fat, as compared with 5%; the fats used included several types of lard, oleo oil, and cottonseed oil. The efficiency of utilization, expressed as gain in weight per 100 cal. of food consumed, was greatest on the low-fat diets, however, indicating that the greater growth on the higher fat diets required a greater proportionate consumption of food. Hajdu ('42) studied the growth of working rats on diets in which fat and carbohydrate contents were varied reciprocally. The best growth, and the best work performance, were observed when the fat and carbohydrate contents of the diet were approximately equal while high-fat and low-fat diets resulted in poorer growth and performance. Crampton and Mills ('45) eliminated the effect of food consumption by feeding rats isocaloric amounts of diets containing 4% and 16% of fat. They report better growth on 4% than on 16%, whether the animals were fed ad libitum or isocalorically. Opposite results were obtained in a carefully planned experiment by Forbes and coworkers ('46a, '46b). They report greater growth with an increasing fat content of the diet at levels of 2, 5, 10 and 30% when isocaloric amounts were fed. The greatest improvement was seen between 2 and 5%. They attribute this effect to increased efficiency of utilization of the diet with a higher fat content.

The experiments to be reported here were designed to examine the question of optimum fat level under conditions of ad libitum feeding and isocaloric feeding at severely restricted levels of food intake. We have studied effects on growth, reproductive performance, physical capacity, nitrogen metabolism, blood composition, and body composition. The studies on nitrogen metabolism, blood composition, and physical capacity have been reported in part elsewhere (Miller et al.,

'46; Scheer et al., '47a). The present paper presents data on growth, reproduction and physical capacity on ad libitum feeding; the results of restricted feeding experiments will appear in succeeding papers (Scheer et al., '47b, '47c).

METHODS

The composition of the diets used is presented in table 1. The rations were based on those of Cerecedo and Vinson ('44).

TABLE 1

Percentage composition of diets used in fat studies.

DIETARY COMPONENT	DIET NUMBER								
	60a	60b	60c	61	62 and 67 [2]	63	64	65	66
Casein	25 [1]	25 [1]	25.5 [1]	27	28	31	37.5	34	39
Sucrose	67	67	65	61	54	40	12	26	0
Fat [3]	0	0	0	4	9	19	39	29	49
Cellulose [4]	3	3	3	3	3.5	4	4.5	4	4.5
Salt mixture [5]	4	4	4	4	4.5	5	6	5.5	6.5
Water-soluble vitamin mixture [6]	0.16	0.16	0.16	0.17	0.18	0.20	0.24	0.22	0.26
Fat-soluble vitamin mixture	1 [7]	1 [8]	1 [8]	1 [9]	1 [9]	1 [9]	1 [9]	1 [9]	1 [9]
Methyl linolate			1.5						
Fat, weight %	0	0	0	5	10	20	40	30	50
Fat, % calories	(2.4)	(2.4)	(5.9)	11	21.5	39	64.5	51.4	69.6
Calories per gm	3.77	3.77	3.84	3.97	4.18	4.64	5.58	5.25	6.06

[1] Vitamin test casein from General Biochemicals, Inc., used. Commercial casein used in other diets.

[2] Diet 67 contains 7.2% of margarine fat and 2.8% cottonseed oil (including 1% in fat-soluble vitamin mixture).

[3] Cottonseed oil (Wesson oil) or margarine fat — the latter being designated by the addition of M to the diet numbers in the text.

[4] Cellu flour obtained from Chicago Dietetic Supply House.

[5] Osborne-Mendel salt mixture.

[6] The water-soluble vitamin mixture had the following percentage composition: choline, 93.50; thiamine chloride, riboflavin and pyridoxine, each 1.24; calcium pantothenate, 2.48; folic acid, 0.30.

[7] The fat-soluble vitamin mixture no. 1 had the following composition per 100 gm; α-tocopherol (Merck), 0.50 gm; carotene (General Biochemicals, Inc.), 20 mg; crystalline vitamin D_2 (Winthrop), 0.5 mg; ethyl laurate (Eastman) to 100 gm.

[8] Same as mixture no. 1 with methyl linolate replacing ethyl laurate.

[9] Same as mixture no. 1 with cottonseed oil replacing ethyl laurate.

Purified or synthetic materials were used because we desired to vary the fat content of the diet without changing the protein, vitamin or mineral intake. The composition of the diets was adjusted so that the ratio of protein, water-soluble vitamins, cellulose, and salts to calories would be the same in all diets. Thus, the intake of each of these components would be the same in isocaloric feeding, and proportional to caloric intake in ad libitum feeding. Ethyl laurate was used as a carrier of the fat-soluble vitamins in the fat-free diet (60a), methyl linolate was provided in diet 60b, and diet 60c was designed to provide a liberal allowance of methyl linolate for rats on restricted feeding. Cottonseed oil [2] was used as a vitamin carrier in all other diets. The margarine fat used in some of the diets contained no milk moisture or milk solids. The diets were mixed fresh at least once weekly in a mechanical dough-mixer. They were kept under refrigeration at all times. For comparison, a stock diet of natural foods was fed to 1 group of animals in some experiments. This diet was composed of ground whole wheat 34%, ground steel-cut oats 34%, skim milk powder 15%, cottonseed oil containing 1600 I.U. vitamin A and 160 I.U. vitamin D, 10%, alfalfa leaf meal 4%, dry yeast (Anheuser-Busch, strain G) 2%, sodium chloride 0.5% and calcium carbonate 0.5%.

Weanling rats from our stock colony were used. The animals were separated from the mothers at 21 days, and divided into experimental groups. Animals from any one litter were assigned to the several dietary groups in regular rotation. In the first experiment, approximately one-half of each litter was assigned to the ad libitum series, and the remainder to the restricted feeding series (Scheer et al., '47b). Each dietary group comprised 50 animals, 25 males and 25 females. The animals on ad libitum feeding were kept in groups of 4 in large cages. Five rats of each sex from each dietary series were placed in smaller individual cages for observations on food consumption. All animals were weighed once weekly.

[2] Wesson oil.

RESULTS

Growth

The growth curves for the males on cottonseed oil and margarine fat diets are plotted in figure 1 and those for the females in figure 2. The body weights fall into 3 rather definite groups in both cases. In the first experiment, the poorest growth was obtained with diets 60a and 60b, containing no

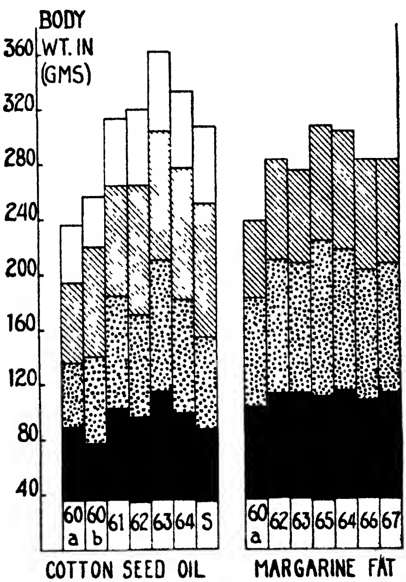


Figure 1

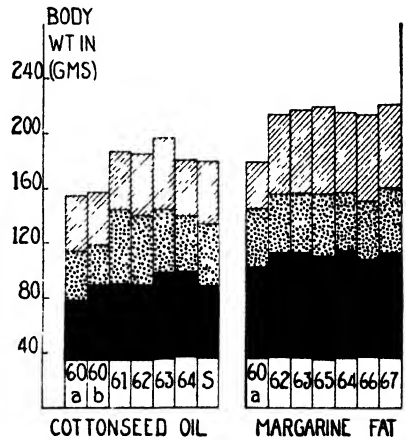


Figure 2

Fig. 1 The average body weight of weanling male rats at start (lower blank space), after 3 weeks (solid black), after 6 weeks (stippled), after 12 weeks (cross-lined) and after 18 weeks (top blank space). The figures in the lower blank space are diet numbers.

Fig. 2 The average body weight of weanling female rats at start (blank space), after 3 weeks (solid black), after 6 weeks (stippled), and after 12 weeks (cross-lined). The figures in the blank space are the diet numbers.

fat. Very much better growth was observed with diets 61, 62, and 64 containing 5, 10 and 40% fat, respectively. The best growth appeared on diet 63, with 20% fat. In the second experiment, growth was poor on 60a, much better on 67M,

62M (10%), 63M (20%) and 66M (50% fat), and best on 64M (40%) and 65M (30%). It is interesting to note that growth on 60a was much better in the second experiment than in the first, while growth on 62M and 64M was better than on 62 and 64, respectively, and on 63M was poorer than on 63. It does not seem possible to explain these latter differences without further experiments. Three conclusions are supported by the 2 experiments: (1) Growth is better on any diet containing fat than on 60a or 60b which had no fat. (2) The optimum fat content for growth lies above 10% and below 50%. (3) There are no consistent differences between diets containing cottonseed oil as compared with margarine fat. Statistical analysis of the data confirms these conclusions (table 2). The most striking differences in the cottonseed oil series are those between 60b and 61, and between 62 and 63. In the margarine series, 60a was decidedly inferior to the other diets while 64M and 65M were superior to diets with less fat. The growth curves for the female rats follow the same pattern (fig. 2 and table 2); diets 60a and 60b were inferior to the others in promoting growth. However, there were no significant differences among the diets containing 5 to 50% fat, as regards growth of females.

It is of interest to compare growth with food consumption on the various diets (table 3). Five rats of each sex were fed each of the experimental diets and were kept in individual cages; records were kept of food consumption. The gains in weight, and total calories consumed are presented in table 3 for the first 6 and 12 weeks of the experiment. In general, it appears that the greatest growth was accompanied by the greatest food consumption, although there are some exceptions among the females. It might accordingly be concluded that the differences in promoting growth in the diets used depend simply on variations in the amounts which rats will consume in ad libitum feeding. We have no positive proof that this is not the whole explanation but Forbes and coworkers ('46a, '46b) found similar differences with isocaloric feeding, and we have noted a superiority of diets containing fat in severely

TABLE 2

The mean weights of male and female rats after 12 weeks on diets containing different amounts of cottonseed oil or margarine.

MALE RATS				FEMALE RATS			
Diet no.	% fat	Average weight [1]	M.D. S E M D [2]	Diet no.	% fat	Average weight [1]	M.D.. S E M.D. [2]
			Cottonseed oil diet				
60a	0	193.7 ± 8.2	60b,2.4; 61,6.5; 62,4.7; 63, 10.5; 64,7.4; S,5.9	60a	0	154.5 ± 4.3	61,6.2; 62,5.3; 63,7.6; 64,6.5; S,4.7
60b	0	219.9 ± 5.9	61,4.9; 62,4.9; 63,5.0; 64, 5.8; S,3.9	60b	0	157.1 ± 4.3	61,5.7; 62,5.0; 63,7.1; 64,6.0; S,4.2
Stock	14	251.0 ± 5.2	63,6.3; 64,2.8	Stock	14	179.7 ± 3.3	63,3.5
61	5	264.1 ± 7.0	63,4.2	61	5	187.0 ± 2.9	63,2.2
62	10	264.3 ± 6.8	63,4.2	62	10	135.4 ± 3.9	63,2.2
64	40	277.6 ± 7.8	63,2.5	64	40	190.3 ± 3.5	
63	20	304.0 ± 5.6		63	20	197.0 ± 3.6	
			Margarine fat diet				
60a	0	238.2 ± 5.1	62,6.6; 63,5.4; 64,10.3; 65, 10.0; 66,6.4; 67,6.7	60a	0	179.4 ± 3.0	62,3.3; 63,3.4; 64,3.5; 65,5.1; 66,3.1; 67,4.9
63	20	274.6 ± 4.4	64,5.0; 65,5.1	66	50	193.5 ± 3.3	
66	50	282.1 ± 4.5	64,3.6; 65,3.8	62	10	194.0 ± 3.2	
62	10	282.5 ± 4.4	64,3.5; 65,3.8	64	40	194.8 ± 3.2	
67	10	282.7 ± 4.2	64,3.6; 65 3.8	63	20	197.0 ± 4.3	
64	40	303.0 ± 3.7		65	30	199.4 ± 2.5	
65	30	306.5 ± 4.5		67	10	201.0 ± 3.9	

[1] Including Standard Error of Mean calculated as follows $\sqrt{\Sigma d^2/n} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

[2] Ratio is given for diets in which growth is significantly greater. In each case the ratio follows the number of the dietary group compared. The figure 2.5 is taken as the lowest significant ratio (only 1 chance in 100 that this is not significant).

TABLE 3

The relation of average gain in weight to food consumption of rats (5 in each group) kept in individual cages for 12 weeks.

DIET NO.	MALE RATS						FEMALE RATS					
	6 weeks			12 weeks			6 weeks			12 weeks		
	Average gain	Total Cal.	Gain/100 Cal	Average gain	Total Cal.	Gain/100 Cal.	Average gain	Total Cal	Gain/100 Cal	Average gain	Total Cal	Gain/100 Cal
	gm			gm			gm			gm		
					Cottonseed oil diet							
60a	72.6	1310	5.54	113.2	3370	3.36	67.0	1320	5.07	98.6	3120	3.06
60b	30.8	880	3.50	139.4	2740	5.09	50.8	1090	4.66	112.2	2940	3.82
61	137.6	1720	8.00	220.4	4020	5.50	100.0	1720	5.80	140.2	3750	3.74
62	134.0	1920	6.97	226.8	4360	5.20	98.2	1600	6.14	138.6	3440	4.02
63	173.6	2260	7.66	250.2	4790	5.24	100.2	2020	4.96	164.6	3820	4.30
64	147.8	1865	7.91	215.0	4230	5.09	104.0	1880	5.54	138.0	3860	3.58
					Margarine fat diet							
60a	139.0	1680	8.27	189.6	3740	5.07	100.0	1420	7.04	134.6	3180	4.23
62	164.6	1780	9.25	241.2	3920	6.15	110.0	1610	6.83	148.0	3280	4.51
67	157.5	1760	8.95	234.4	3820	6.15	113.8	1430	7.95	154.0	3040	5.06
63	174.0	1890	9.20	239.0	4020	5.95	118.8	1740	6.82	167.2	3540	4.74
65	179.2	2000	8.96	267.4	4360	6.12	112.0	1750	6.40	150.4	3590	4.19
64	160.0	1630	9.80	243.4	3770	6.19	118.2	1630	7.26	153.8	3250	4.72
66	158.0	1745	9.06	245.6	3840	6.37	103.8	1680	6.16	142.8	3140	4.14

restricted isocaloric feeding (Scheer et al., '47b, '47c). In this connection, it should be noted that the efficiency of utilization of the diets, as expressed by the gain in weight per 100 cal. of food consumed, varies only slightly, and in no consistent fashion among the diets. There are instances where the efficiency is lowest when the growth is least, but this is not invariably the case. The efficiency is greater in the males than in the females and in general the highest efficiency obtains for the period of rapid growth (first 6 weeks). These data are in agreement with our earlier results (Deuel et al., '44).

Physical capacity

The methods used for the determination of physical capacity and some of the results obtained have been reported elsewhere (Scheer et al., '47a). The method depends on a measurement of the time required for a rat to become exhausted when swimming with a regularly increasing work load. The results for the present experiments are summarized in table 4. Statistical analysis of these data shows the

TABLE 4

The physical capacity of rats fed diets containing different amounts of fat ad libitum.

SEX	WEEKS ON DIET	DURATION OF SWIM IN SECONDS									
		60a 0%	60b 0%	61 5%	62 10%	63 20%	65 30%	64 40%	66 50%	Stock 14%	67[1] 10%
		Cottonseed oil as the dietary fat									
♀	6	512	580	802	777	1062		922		1025	
		(5)	(5)	(6)	(5)	(5)		(3)		(4)	
♂	6	805	692	1019	921	1223		1143		955	
		(5)	(5)	(5)	(5)	(5)		(5)		(4)	
♂	12	645	637	769	850	846		1068		1084	
		(20)	(19)	(19)	(17)	(19)		(15)		(19)	
		Margarine fat as the dietary fat									
♂	12	485			806	1174	1065	876	1122		1218
		(23)			(10)	(8)	(25)	(4)	(10)		(8)

Figures in parentheses are number of tests.

[1] 7.8% margarine fat and 2.2% cottonseed oil.

following significant differences for the males (mean difference/standard error greater than 2.5). In the first series (cottonseed oil) tested after 6 weeks, diet 63 was superior to 60a and 60b, and 61 was superior to 60b. After 12 weeks physical capacity was greater on diets 62, 64 and the stock diet than on 60a or 60b, while the stock diet was also superior to 61 and 62. In the second experiment (margarine fat), capacity was greater on 62M, 63M, 65M, 66M than on 60a, and greater on 63M and 67M than on 62M. These data do not present a wholly consistent picture, but it is evident that, in all cases, physical capacity is low on diets containing no fat, as compared with diets containing 10 to 50% fat. It is of particular interest that the stock diet, which does not support growth as well as diets 62 and 63, is superior to diet 62, and probably to 63, in the development of physical capacity. It is likewise of interest that diet 67M, which differs from 62M only in the presence of a small amount of cottonseed oil in place of part of the margarine fat, and which supports almost exactly the same amount of growth, is apparently superior in providing for development of physical capacity. It is unfortunate that so few animals were tested in this group.

Reproductive performance

The data on reproduction are summarized in table 5. The females in the cottonseed oil series were checked regularly for sexual maturity, as indicated by opening of the vagina. The table shows that maturity was attained more rapidly on the diets (61–64 and stock) containing fat than on the fat-free diet. After 16 weeks on the experimental diets, 8–10 females from each dietary group were mated with males from the same group. The litters were reduced to 7 animals each at 3 days after birth. Fertility, as indicated by the percentage of matings which resulted in pregnancy and birth, was lowest on diet 60b, where no pregnancies developed, and relatively low on 60a, where only 2 litters were born. The animals born to mothers on diet 60a were smaller when weighed 3 days after birth, and remained smaller during the lactation period than

those born to mothers on diets containing fat; the fat-free diet evidently does not support adequate lactation. Considerable variation in fertility and per cent mortality of the young rats was evident among the animals receiving the diets containing fat, but there was no consistent relation to fat content of the diet. Breeding tests were not performed with the animals in the second (Margarine fat) series.

TABLE 5

Reproductive performance of rats fed experimental diets. Bred 16 weeks from beginning of experiment.

DIET	AGE OF FEMALES AT MATURITY (WEEKS)	NO. OF MATINGS	% OF MATINGS SUCCESSFUL	AVERAGE TIME, DAYS, FROM MATING TO BIRTH	AVERAGE NO. OF ANIMALS PER LITTER	AVERAGE WEIGHT OF			% MORTALITY BEFORE 21 DAYS
						Litter at 3 days	Individuals		
							at 3 days	at 21 days	
60a	8.1	9	22.2	36.0	6.0	36.5	6.1	17.3	0
60b	9.1	9	0						
61	7.0	8	62.5	25.6	8.0	67.6	8.5	36.9	10
62	6.5	10	90.0	26.8	6.5	55.0	7.6	27.3	49
63	6.4	9	55.6	26.2	6.2	51.0	8.3	33.1	13
64	6.3	10	70.0	30.7	8.0	58.0	7.9	32.4	30
Stock	6.6	10	90.0	31.0	7.8	66.7	7.6	30.7	23

DISCUSSION

Where we have noted significant differences in performance related to the fat content of the diet, these have all been in the same direction; animals receiving diets containing 5 to 50% fat grow more rapidly, reach a greater final weight, have a greater capacity for exhausting work, attain sexual maturity earlier, are more fertile, and raise larger young than wholly comparable animals fed diets containing no fat. This was not the result of fatty acid deficiency, or deficiency of fat-soluble vitamins; the diet (60b) with added methyl linolate gave just as poor results as the diet (60a) containing only ethyl laurate. Growth was better on diets containing intermediate amounts of fat (20–30%) than on similar diets with small (5–10%) or large (40–50%) amounts. The nature of the fat, as between

cottonseed oil and margarine fat (hydrogenated vegetable oils), had little consistent influence on the results.

These conclusions are in agreement with those reached by Hoagland and Snider ('40) and by Forbes et al. ('46a, '46b). At least 2 factors are apparently involved in the superiority of diets containing fat, although our data do not permit an evaluation of the relative importance of these factors, or an estimate of other possible influences. The first factor is the greater intake in terms of energy values of diets containing fats. This was noted by Hoagland and Snider ('40) and is evident in our results. A second component influence is the greater efficiency of utilization reported by Forbes et al. ('46a, '46b) with diets containing larger amounts of fat. They found that the energy cost of metabolizing diets providing constant protein intake decreased with increasing ratio of fat to carbohydrate. Our data, however, provide evidence of an optimum fat intake somewhere between 30 and 50% of the diet (50–70% of total cal.) at least as regards growth. There is no evidence that the low-fat or fat-free diets give more efficient food-utilization as indicated by Hoagland and Snider ('40). In fact, the lowest efficiency (gain in weight/100 cal.) was obtained on the fat-free diet in all cases.

It appears from the tests of reproductive performance and physical capacity that factors other than fat content as such may be important. Particularly noteworthy is the superior performance of the stock diet in supporting exhausting work, as compared with synthetic diets of approximately the same fat content. It appears desirable to examine this question further, with numbers of animals sufficiently great to offset the variability inherent in the determination.

We originally planned to observe the effects of our experimental diets on longevity. It has proved impossible thus far to do this, but the results of such a study would be of particular interest; the more rapid growth observed on diets containing fat might well be associated with a shortened life span. It seems also of value to note that growth remains the most sensitive criterion among those which we have used in esti-

mating the effects of these diets; whether it is the most valid is more questionable.

SUMMARY

Ad libitum feeding to rats of diets varying in fat content from 5 to 50% (10 to 70% of total cal.) results in better growth, greater physical capacity, and better reproductive and lactation performance in rats than does feeding of diets containing minimal amounts of fat. The difference is not influenced by presence of methyl linolate in the fat-free diet. Optimum growth was observed on diets containing 20–40% fat. The most pronounced differences were observed in males. The effects are in part, but not entirely, attributable to greater caloric intake on the diets containing fat.

LITERATURE CITED

BROWN, W. R., A. E. HANSEN, G. O. BURR AND I. MCQUARRIE 1938 Effects of prolonged use of extremely low-fat diet on an adult human subject. J. Nutrition, *16:* 511.

BURR, G. O. 1942 Significance of the essential fatty acids. Fed. Proc., *1:* 224.

CERECEDO, L. R., AND L. J. VINSON 1944 The effect of the L. casei factor and of a highly potent folic acid concentrate on lactation in rats maintained on synthetic rations. Arch. Biochem., *5:* 469.

CRAMPTON, E. W., AND M. F. MILLS 1945 The effect of heating, the presence of antioxidants, and the level and melting point of the fat component on the nutritional value of diets as indicated by rat feeding experiments. Canadian J. Research Series E, Medical Sciences, *23:* 131.

DEUEL, H. J., JR., E. MOVITT, L. H. HALLMAN AND F. MATTSON 1944 Studies on the comparative nutritive value of fats. I. Growth rate and efficiency of conversion of various diets to tissue. J. Nutrition, *27:* 107.

FINNERUD, C. W., R. L. KESLER AND H. F. WIESE 1941 Ingestion of lard in the treatment of eczema and allied dermatoses. Arch. Derm. Syph., *44:* 849.

FORBES, E. B., R. W. SWIFT, R. F. ELLIOTT AND W. H. JAMES 1946a Relation of fat to economy of food utilization. I. By the growing albino rat. J. Nutrition, *31:* 203.

——— 1946b Relation of fat to economy of food utilization. II. By the mature albino rat. J. Nutrition, *31:* 213.

HAJDU, I. 1942 Über den Einfluss körperlicher Arbeit auf das Wachstum and Arbeitsvermögen weisser Ratten, den Glykogen- und Fettgehalt ihrer Leber und ihres Muskels, das Gewicht ihrer Nebennieren bei unterschiedlicher kohlehydrat- und fetthaltiger Diät. Pflügers Arch ges. Physiol., *245:* 556.

HANSEN, A. E. 1933 Serum lipid changes and therapeutic effects of various oils in infantile eczema. Proc. Soc. Exp. Biol. Med., *31:* 160.

HIGGINS, H. L. 1930 Some physiological and clinical effects of high fat feeding. New England J. Med., *203:* 145.

HOAGLAND, R., AND C. G. SNIDER 1940 Nutritive properties of certain animal and vegetable fats. U. S. Dept. Agric. Tech. Bull. no. 725.

LOEWY, A., L. W. FREEMEN, A. MARCHELLO AND V. JOHNSON 1943 Increased erythrocyte destruction on a high fat diet. Am. J. Physiol., *138:* 230.

MILLER, Z. B., M. FRIEDMAN AND H. J. DEUEL, JR. 1946 The influence of caloric restriction on plasma protein, hemoglobin and hematocrit values in the rat. Am. J. Physiol., *147:* 423.

SAKO, W. S. 1942 Resistance to infection as affected by variations in the proportions of protein, fat and carbohydrate in the diet. J. Pediatrics, *20:* 475.

SCHEER, B. T., J. F. CODIE AND H. J. DEUEL, JR. 1947c The effect of fat level of the diet on general nutrition. III. Weight loss, mortality and recovery in young adult rats maintained on restricted calories. J. Nutrition (in press).

SCHEER, B. T., S. DORST, J. F. CODIE AND D. F. SOULE 1947a Physical fitness in rats in relation to the energy and fat content of the diet. Am. J. Physiol. (In press.)

SCHEER, B. T, D. F. SOULE, M. FIELDS AND H. J. DEUEL, JR. 1947b The effect of fat level of the diet on general nutrition. II. Growth, mortality and recovery in weanling rats maintained on restricted calories. J. Nutrition, *33:* 583.

THE EFFECT OF FAT LEVEL OF THE DIET ON GENERAL NUTRITION

II. GROWTH, MORTALITY AND RECOVERY IN WEANLING RATS MAINTAINED ON RESTRICTED CALORIES [1]

BRADLEY T. SCHEER, DOROTHY F. SOULE, MAX FIELDS AND H. J. DEUEL, JR.

Department of Biochemistry and Nutrition, University of Southern California School of Medicine, Los Angeles

ONE FIGURE

(Received for publication November 27, 1946)

Interest in phenomena related to undernutrition has been intensified in recent years by widespread conditions of famine. The studies of Keys and coworkers ('46) with typical famine diets have contributed valuable information concerning the effects of starvation on man. We wished to examine in more detail the effects on rats of variations in the fat content of purified diets under severe caloric restriction.

These experiments to be reported here were carried on simultaneously with those reported in the preceding paper of this series (Deuel et al., '47). Litter mates were divided between the experiments, the same diets were fed, and the same measurements of growth, reproductive performance and physical capacity were used.

[1] The subject matter of this paper has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

We are indebted to Merck and Co., Rahway, N. J., for the synthetic B vitamins; to the Winthrop Chemical Co., Albany, N. Y., for the crystalline vitamin D_3 and to Lederle Laboratories, Inc., for the folic acid.

METHODS

The diets were designed so that the ratio of protein, vitamins or minerals to calories was the same in all diets. Thus, when the caloric intake was held constant the only variation among animals on different diets was in the intake of fat and carbohydrate; the intake of vitamins, proteins and minerals remained constant.

The animals were kept singly in especially designed cages ($7 \times 8 \times 6$ inches) in a room at constant temperature of 78° during the period of caloric restriction. On transfer to ad libitum feeding, the animals were kept in groups of 4 in larger cages ($16 \times 10 \times 11$ inches).

The animals on restricted calories were fed the diets on alternate days, at the same hour each day, in weighed portions. It was realized that this practice, necessitated by shortage of personnel, would influence to some extent the results of the experiment. However, we considered that comparisons between groups receiving different diets would be valid, since all groups were being dealt with in the same way. The animals were weighed once weekly.

RESULTS

Caloric restriction at various levels

In our first experiment, we wished to establish the lowest level of caloric intake which would permit survival during 12 weeks in weanling rats. Male rats 21 and 28 days old were used. They were fed the stock diet at levels ranging from 13 to 35 Cal. per day for 12 weeks. The general results are presented in table 1. The most striking feature of the results is the fact that the final weight attained depends on the caloric intake per day, regardless of the initial weight or age of the animal. The relation is linear and may be expressed as

$$W = aC - K$$

where W is the final weight in gm and C the caloric intake per day. The constants in this case have the values, $a = 8.25$ and $K = 47$.

TABLE 1

Weight changes in male weanling rats fed stock diet at varying levels of caloric intake for 12 weeks.

CALORIES FED PER ANIMAL PER DAY	NUMBER OF ANIMALS	AVERAGE WEIGHT		INITIAL AGE
		Initial	Final	
		gm	*gm*	*days*
34.7	9	55.4	217	28
27.7	8	46.2	180	28
24.1	7	39.7	142	21
23.1	9	60.4	142	28
19.1	7	42.8	112	21
18.8	9	57.5	106	28
17.1	7	40.8	95.5	21
14.7	7	38.7	74.5	21
14.5	10	56.2	72.7	28
12.9	7	39.5	59.9	21

Caloric restriction with variable fat content in the diet

On the basis of this preliminary work, it was concluded that weanling rats 21 days of age could survive for 12 weeks on a diet providing 12 Cal. per day. Accordingly, 300 animals, litter mates of those used by Deuel et al. ('47), were distributed among dietary groups, 25 males and 25 females in each group. They were placed in individual cages when 21 days old, and fed the experimental diets (60c, 61, 62, 63, 64, and stock) in weighed portions to provide 24 Cal. every second day. A few of the animals failed to consume their food and died within a day or 2 after transfer to the experimental cages; these were replaced, but data from them were not included in the records reported here.

The gains in weight on the different diets are presented in table 2. The standard errors of the mean weights range from $\pm$ 1.0 to 1.5. The standard error of a difference between 2 means would then be about $\pm$ 2.0. There is accordingly about 1 chance in 100 that a difference in weight as great as 5 gm could arise by chance. If a difference of 5 gm is then taken as significant, we may say that diets containing fat permit

better growth on severely restricted calories than does diet 60b, lacking in fat but containing adequate amounts of linoleic acid and the fat-soluble vitamins. The increase in weight is, of course, very small as compared with growth on the same diets fed ad libitum (Deuel et al., '47).

The mortality figures in table 2 represent the number of animals which died after 2 or more weeks on the experimental diets. It is remarkable that the greatest number of deaths

TABLE 2

Total average gains in weight (gm) of rats fed experimental diets at a level of 12 cal. per day for 12 weeks after weaning.

DIET	% FAT	INITIAL WEIGHT	GAIN IN WEIGHT AFTER				NO. OF DEATHS
			3 weeks	6 weeks	9 weeks	12 weeks	
			Males				
60c	0[1]	36.4	3.2	7.1	10.1	18.5	4
61	5	33.9	6.1	13.3	14.9	25.1	8
62	10	36.1	5.9	10.8	12.0	19.7	5
63	20	35.8	7.1	16.6	15.7	20.0	4
64	40	36.6	6.7	14.6	16.7	25.6	3
Stock	14	35.4	5.6	12.9	13.7	21.1	2
			Females				
60c	0[1]	36.4	2.0	4.7	6.3	15.3	5
61	5	36.1	0.1	4.7	10.5	20.9	17
62	10	34.3	4.7	8.9	10.7	19.5	9
63	20	36.1	6.8	11.1	11.4	21.0	4
64	40	35.1	6.6	11.0	12.9	21.7	3
Stock	14	36.8	3.0	9.9	10.0	16.5	2

[1] Contains 2.5% methyl linolate.

occurred on diets 61 or 62, with less on diets 60c and 63, still less on 64, and the least on the stock diet. Most of the deaths occurred 3 to 4 weeks after the beginning of the experiment.

Cottonseed oil, the fat used in these diets, contains about 40% linoleic acid (Hilditch, '40). Diets 60c and 61 therefore have approximately the same content of linoleic acid, but in the former it is present as the methyl ester, and in the latter as glycerides with other fatty acids. It is difficult to account for the observed mortality in the basis of this difference

in composition. Animals fed these diets ad libitum showed no mortality.

It is possible that the observed mortality could have resulted from drafts in the animal room, since the animals on different diets were grouped in different racks of cages. However, the animals on 60c and 61 were in the same cage rack, and the room was small and ventilated by a forced air draft, so this explanation seems unlikely. Death in some cases was associated with pulmonary inflammation and hemorrhage from the nostrils. In other cases, these symptoms were absent.

Recovery from the period of caloric restriction

Weight. At the end of the 12-week period of restricted caloric intake, the animals were placed on ad libitum feeding on the same diets, except that diet 60c was replaced by 60b containing 1% methyl linolate instead of 2.5%. Record was kept of the weight of the males for 12 weeks, but females were weighed only for 3 weeks after transfer (fig. 1). After 3 weeks, breeding tests were started with most of the females, and the weights would consequently not have represented merely growth changes. In general, the variations in weight between animals on different diets were small, but statistically significant differences (difference/standard error >2.5) were apparent between diet 60b containing no fat but with the addition of methyl linolate, and diets 62 to 64 containing 10 to 40% fat. Growth on the stock diet, with 14% fat was also inferior in some cases to growth on diets 62 to 64, but this may perhaps in part be attributed to the lower protein content of the stock diet (protein 15% of stock diet, 25 to 37% in diets 60 to 64).

Moreover, rapid growth was still occurring during the 9- to 12-week period after ad libitum feeding was instituted in the rats receiving diets which contained 10% or more fat while it had practically stopped in the rats receiving the low-fat diets. The following increments of growth were noted for the 9- to 12-week period: 60b, 4.3 gm; 61, 6.6 gm; 62, 17.3 gm; 63, 14.6 gm; 64, 27.1 gm and stock diet, 26.0 gm.

Maturity of females. Regular observations were made to determine the time of opening of the vagina in the female rats as a criterion of sexual maturity. None of the females became mature during the 12-week period of restricted feeding; controls fed the same diets ad libitum matured within 3 to 6 weeks after weaning (Deuel et al., '47). However, the vagina opened very shortly after transfer from restricted to ad libitum feeding. Maturity was evident earlier in rats fed the diets containing fat, as compared with the fat-free diet (table 3).

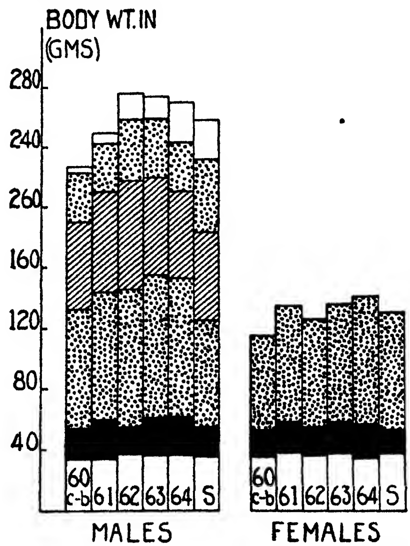


Fig. 1 The average body weight of weanling rats at start (lower blank space), after 12 weeks on restricted calories (solid black), after 3 weeks ad libitum (stippled), after 6 weeks ad libitum (lined), after 9 weeks ad libitum (upper stippled), and after 12 weeks (upper blank space). The figures in the lower blank space are the diet numbers.

TABLE 3

Time (weeks) from transfer to ad libitum feeding to sexual maturity (opening of vagina) in female rats fed restricted amounts of diets varying in fat content for 12 weeks after weaning.

DIET	60b	61	62	63	64	STOCK
Fat content in %	0	.5	10	20	40	14
Time to maturity, in weeks	2.1	1.7	1.5	1.1	1.4	1.2

TABLE 4

Reproductive performance of female rats recovering from a period of undernutrition.

DIET	FAT CONTENT	MATED WITH MALES FROM	NO. OF MATINGS	% OF MATINGS SUCCESSFUL	AVERAGE TIME FROM BIRTH TO MATING	AVERAGE NUMBER OF ANIMALS PER LITTER		AVERAGE LITTER WEIGHT AT		WEIGHT OF INDIVIDUALS AT		MORTALITY BEFORE 21 DAYS
								3 days	21 days	3 days	21 days	
	%				days			gm	gm	gm	gm	%
60b	0 [1]	60b	6	67	28	5.3						
							5.3	47.6	152	8.9	26.1	9
		N [2]	2	100	24	5.5						
61	5	61	3	100	25	8.3						
							6.8	47.7	219	7.1	32.4	0
		N	1	100	29	2.0						
62	10	62	4	100	31	8.3						
							7.7	64.3	167	8.4	27.8	35
		N	2	100	25	7.5						
Stock	14	Stock	5	60	34	6.0						
							7.0	53.8	175	7.7	23.3	14
		N	2	100	24	8.5						
63	20	63	5	100	26	7.6						
							8.5	60.8	230	7.2	31.8	12
		N	5	80	24	9.5						
64	40	64	5	80	30	9.5						
							8.9	72.3	283	8.1	30.6	8
		N	5	80	28	7.8						

[1] Contains 1% methyl linolate.

[2] N signifies males from stock colony.

Fertility and lactation. Breeding tests were initiated 5 to 6 weeks after transfer to ad libitum feeding. Five females from each dietary group were mated with males from the same group; litter mates were not mated. Five females from each group were also mated with males from the stock colony. In some instances, not enough females were available, and the number of matings was less than 10. The litters were not reduced in size after birth; they were weighed at 3 and 21 days. By allowing the female to keep the full litter, we felt that this would offer a more critical test of the lactation capacity than in the first experiment (Deuel et al., '47) where the litters were reduced to 7. A mating was considered unsuccessful if pregnancy had not developed within 2 months. The results of these tests are summarized in table 4.

Fertility was not greatly reduced except on the stock diet, and on diet 60b. Here only 3 out of 5, and 4 out of 6 matings, respectively, were successful when males from the same dietary group were used. When males from the stock colony (N) were used in 2 matings in each case, both were successful. It is regrettable that we were unable to make more matings with stock colony males, since it appears that the lowered fertility in these cases may be due to the males. The length of the period from the time at which the rats were placed together in the cage to the birth of the litter shows no consistent variations, except that the period was in general shorter when stock colony males were used.

The number of animals per litter, and the weight of the litter at 3 and 21 days, shows an increase with increasing fat content of the diet. The greatest difference is that between diets 60b and 61; diets 63 and 64 appear to be superior to the others in supporting lactation.

DISCUSSION

The results of this study are in agreement with other work from this laboratory: the work capacity of rats (Scheer et al.,

'47), growth on ad libitum feeding (Deuel et al., '47), ability to withstand a prolonged period of undernutrition, growth during recovery from such a period, rate of sexual development in females, fertility, and lactation performance, are all greater on diets containing liberal amounts of fat (10 to 40%) than on diets containing little or no fat (0 to 5%). The optimum fat level for growth appears to lie between 20 and 40% (Deuel et al., '47), but there is some evidence that work capacity (Scheer et al., '47) and fertility are higher on diets containing 40% fat. It is interesting to compare the data on reproduction with those presented by Deuel et al. ('47) for litter mates of the animals of the present experiment, fed ad libitum. Particularly noteworthy is the difference in fertility on the fat-free diet; the animals on ad libitum feeding had no offspring while 75% of matings in the present experiment were successful. The litters produced by the females which had recovered from the period of restricted feeding were not smaller, and in some cases were larger than those produced by females fed ad libitum.

SUMMARY

During a period of severely restricted feedings, weanling rats receiving isocaloric amounts of diets varying in fat content grow better on diets containing fat than on a similar diet lacking fat. Mortality, from uncertain causes, was highest on the diet with 5% fat. During a recovery period of ad libitum feeding, growth, fertility, and lactation were better supported by diets with liberal amounts of fat than by a fat-free diet.

LITERATURE CITED

Deuel, H. J., Jr., E. R. Meserve, E. Straub, C. Hendrick and B. T. Scheer 1947 The effect of fat level of the diet on general nutrition I. Growth, reproduction and physical capacity of rats receiving diets containing various levels of cottonseed oil or margarine fat ad libitum. J. Nutrition, *33:* 569.

Hilditch, T. P. 1940 The Chemical Constitution of the Natural Fats. John Wiley and Sons, New York.

Keys, A. 1946 Human starvation and its consequences. J. Am. Dietetic Assoc., *22:* 582.

Scheer, B. T., S. Dorst, J. F. Codie and D. F. Soule 1947 Physical fitness in rats in relation to the energy and fat content of the diet. Am. J. Physiol. (In press.)

THE NET PROTEIN VALUE OF FOOD YEAST

JOSÉ A. GOYCO AND CONRADO F. ASENJO
Department of Chemistry, School of Tropical Medicine,
San Juan, Puerto Rico

(Received for publication November 25, 1946)

The concept of net protein value was proposed by Mitchell and Carman ('24) to appraise, in a single figure, the value of a food as a source of dietary protein. Such a figure combines the biological value of the protein, its coefficient of digestibility, and also its original content of "conventional" or, so-called, crude protein — i.e., N × 6.25.

Very few investigations are found in the literature dealing with the determination of the coefficient of digestibility and the biological value of yeast protein using the nitrogen metabolism technic proposed by Thomas ('09) and later developed and adapted to growing rats by Mitchell ('24a, '24b, '24c) and Mitchell and Carman ('26). This biological assay procedure is considered to give the most satisfactory picture of protein utilization by the body. Mitchell ('24b) fed an undescribed type of yeast at a level of 5% protein in the diet and recorded a coefficient of digestibility of 77 and a biological value of 85.5. Still and Koch ('28) assayed a sample of dried bakers' yeast and obtained a coefficient of digestibility of 72.06 and a biological value of 25.2. Their diet contained 38% yeast, which was equivalent to about 20% crude protein. No doubt the low biological value obtained for bakers' yeast by Still and Koch was due, at least in part, to the high level of yeast protein in the diet. As no information was given in either of these studies, with respect to the protein (N × 6.25) content of the yeast samples investigated, it is impossible to

use these data to compute the respective net protein values. Recently, Hughes and Hauge ('45) determined the true digestible coefficient and biological value of dried brewers' yeast and reported values of 83 and 61, respectively. Their diet contained 10% protein (N $\times$ 6.25). The yeast used by them contained about 48.7% protein. The net protein value for brewers' yeast, according to these figures, is 24.7.

Since food yeast might become an important item in the diet of tropical countries the determination of the net protein value of this yeast appeared desirable. To obtain this value, the biological values and the true coefficient of digestibility of the different yeasts were investigated by the method of Mitchell and Carman ('26). Two samples of food yeast were used. One sample was obtained from the Microbiological Section of the Chemical Research Laboratories at Teddington, England (*Torula utilis* no. 1084),[1] and the other was produced at the pilot plant of the Puerto Rico Industrial Development Co. at Río Piedras, Puerto Rico (*Torula utilis* no. 3).[2,3] In the case of the latter, Puerto Rican sugar cane molasses was used in the growing mash. For purposes of comparison, the net protein value of a sample of dried brewers' yeast[4] was also determined.

EXPERIMENTAL PROCEDURE

In general, the technic recommended by Mitchell and Carman ('26) has been followed. Ten male Wistar albino rats of about 50–60 gm body weight were used in each trial. Metabolism cages of the type described by Still and Koch ('28) were used. This type of cage permitted the accurate collection of urine and feces samples separately. The tests were of 10 days' duration divided into 2 periods, a 3-day preliminary period, during which no samples were collected, and a 7-day experimental period during which urine and feces

[1] Supplied through the courtesy of Dr. A. C. Thaysen.

[2] Supplied through the courtesy of Mr. Rafael Fernández-García.

[3] Strain no. 3 obtained from the stock cultures of the University of Wisconsin.

[4] Fleischmann type 2019.

samples were collected daily. Ferric oxide was used as a feces marker for the accurate separation of these 2 periods. Body weights were taken at the beginning and at the end of each balance period. The food offered and residue left were accurately weighed in an analytical balance to determine the food consumed during the 7-day experimental period. The feces were collected daily and dried in an oven to constant weight at 100°C. The entire dried sample for the 7-day period was digested and aliquot samples taken for total nitrogen determination. For the accurate collection of all the voided urine, the cages were thoroughly washed daily with a hot 0.5% sulfuric acid solution. The urine plus the washings were filtered into a 2-liter bottle and kept in the ice-box with xylol as preservative. At the end of the 7-day period, these samples were well mixed and diluted to volume. Aliquot portions were taken for total nitrogen determinations. All nitrogen determinations were made according to the Kjeldahl-Gunning-Arnold method in the Official and Tentative Methods of Analysis ('35).

The 3 yeast diets were made to contain about 8% protein. In the initial and final standardizing periods, to determine the excretion of body nitrogen in feces and in urine, a dried, fat-free whole egg diet of about 4% protein was used. The experimental rations had the composition shown in table 1.

To prevent the scattering of the diet from the food cups, enough cold, distilled water was added to the amount of ration previously weighed to obtain a soft paste. With very few exceptions, this procedure completely prevented the scattering and loss of food.

RESULTS

The results of the initial and final standardizing periods are given in table 2. The purpose of these periods was to determine the excretion of the so-called "metabolic nitrogen" in the feces and the endogenous nitrogen in the urine, the former being dependent upon the dry matter consumed and the latter, upon the body weight of the rat. These factors differ some-

what in the 2 periods for each rat and in using them for the calculation of the biological values for the intermediate experimental periods, they are assumed to change in a linear fashion from the initial to the final value determination. The

TABLE 1

Composition of diets.

CONSTITUENTS:[1]	STANDARDIZING DIET	DRIED FOOD YEAST *T. utilis* No. 1084	DRIED FOOD YEAST *T. utilis* No. 3	DRIED BREWERS' YEAST
	%	%	%	%
Dried, ether-extracted whole egg	6.15			
Dried yeast		16.25	15.78	15.74
Modified Osborne and Mendel salts	3.00	3.00	3.00	3.00
Sucrose	10.00	10.00	10.00	10.00
Sodium chloride	1.00	1.00	1.00	1.00
Vegetable oil (Mazola)	9.00	9 00	9.00	9.00
Cod liver oil	1.00	1.00	1.00	1.00
Corn starch	66.07	56 75	57.22	57.26
Cellu-flour	3.00	3 00	3.00	3.00
Protein content	4.31	8.16	8.19	8.06

[1] Synthetic vitamins added as supplement to each kilo of diet: thiamine, 2 mg; pyridoxine, 2 mg; riboflavin, 8 mg.

TABLE 2

Initial and final standardizing periods. Daily excretion of metabolic and endogenous nitrogen.

	BODY WEIGHT Initial	BODY WEIGHT Final	FOOD INTAKE	FECAL NITROGEN	METABOLIC NITROGEN IN FECES PER GM OF FOOD	URINARY NITROGEN	ENDOGENOUS NITROGEN IN URINE PER 100 GM WEIGHT
	gm	*gm*	*gm*	*mg*	*mg*	*mg*	*mg*
Initial period: dried egg protein ration containing 0.74% N.							
Average of 10 rats	62.1	69.8	5.9	9.2	1.6	16.6	25.3
Standard deviation	3.3	4.0	0.4	0.5	0.2	2.3	5.5
Final period: dried egg protein ration containing 0.69% N.							
Average of 10 rats	89.4	97.9	6.2	9.7	1.5	15.4	16.5
Standard deviation	7.6	6.4	0.7	0.4	0.2	2.2	2.1

average excretion of metabolic nitrogen in the feces was 1.6 mg and 1.5 mg per gm of food consumed in the 2 periods. The endogenous nitrogen output in the urine per 100 gm body weight averaged 25.3 mg in the first standardizing period and 16.5 mg, in the second.

TABLE 8

Nitrogen metabolism of rats on yeast rations.
(Results expressed on the daily basis.)

	BODY WEIGHT		FOOD INTAKE	NITROGEN		
	Initial	Final		Intake	In feces	In urine
	gm	*gm*	*gm*	*mg*	*mg*	*mg*
	Dried food yeast *(T. utilis)* No. 1084 ration					
Average of 10 animals	68.9	68.9	4.32	61.6	13.8	42.7
Standard deviation	5.7	4.5	0.69	10.0	2.2	7.1
	Dried food yeast *(T. utilis)* No. 3 ration					
Average of 10 animals	70.2	69.0	4.07	58.1	13.6	43.0
Standard deviation	3.8	5.4	0.68	10.1	3.1	5.2
	Dried brewers' yeast ration					
Average of 10 animals	75.1	82.9	5.71	81.5	20.6	36.0
Standard deviation	6.8	6.3	0.60	12.6	2.5	6.0

All of the nitrogen metabolism data collected in the different trials are assembled in table 3. The net protein values, digestion coefficients and biological values computed from these data are summarized in table 4. The method of calculating the values has been fully explained by Mitchell ('24a, b) and by Mitchell and Carman ('26).

TABLE 4

Coefficient of true digestibility, biological value and net protein value of the nitrogenous substances present in yeast.

	DRIED FOOD YEAST *T. utilis* No. 1084	DRIED FOOD YEAST *T. utilis* No 3	DRIED BREWERS' YEAST
(a) % Protein (N × 6.25) in the yeast samples	49.25	50.69	50.81
(b) Total fecal nitrogen [1]	13.8	13.6	20.6
(c) "Metabolic nitrogen" in feces [1]	6.6	6.2	8.8
(d) Food nitrogen in feces (b–c)[1]	7.2	7.4	11.8
(e) Total nitrogen intake [1]	61.6	58.1	81.5
(f) Nitrogen absorbed (e–d)[1]	54.4	50.7	69.7
(g) Total urinary nitrogen [1]	42.7	43.0	36.0
(h) Endogenous nitrogen in urine [1]	14.9	15.3	14.6
(i) Food nitrogen in urine (g–h)[1]	27.8	27.7	21.4
(j) Food nitrogen retained (f–i)[1]	26.6	23.0	48.3
(k) Biological value: $\frac{j}{f} \times 100$	48.8	45.3	69.3
(l) True digestibility coefficient: $\frac{f}{e} \times 100$	88.3	87.3	85.5
(m) Net protein value (a × k × l)	21.2	20.0	30.1

[1] Results expressed in milligrams of nitrogen per day. Values are averages of 10 animals.

DISCUSSION

The true coefficients of digestibility of the 3 yeasts investigated were computed by assessing the metabolic nitrogen in the feces. The true digestibilities thus obtained were as follows: 85.5 for brewers' yeast, 88.3 for *Torula* yeast no. 1084, and 87.3 for *Torula* yeast no. 3.

The nitrogen metabolism data indicate a much better utilization of the absorbed nitrogen derived from the brewers' yeast than of that derived from either one of the *Torula* samples. The average biological values computed from these data were 69.3 for brewers' yeast and 48.8 and 45.3, respectively, for the *Torula* samples no. 1084 and no. 3. This dif-

ference in biological value of the brewers' and *Torula* yeast is noteworthy. It might be ascribed to several variable factors resulting from the difference in species, the nutrients used in growing these organisms and the methods of manufacturing. Some of these factors are the content of protein and non-protein nitrogen in the yeasts, the amino acid make-up of their individual proteins, the availability of these amino acids, etc. In regard to this last factor, it is well known that in the case of soy bean, heat treatment improves the nutritive value of the raw product by increasing the availability of cystine (Mitchell et al., '45). Recently, Axtmayer ('46) has claimed that autoclaving improves the nutritive value of yeast. It has been known for some time that, although yeast protein contains about 2% methionine, apparently, this is not in a readily available form. Perhaps the processing to which a specific yeast is subjected during the recovery and drying operations in the factory may modify its final nutritive value.

In considering the biological value of yeast protein ($N \times 6.25$), it should always be remembered that an appreciable amount of the yeast nitrogen (about 12 to 20%) is in the form of non-protein nitrogen. This should not constitute an argument against evaluating yeast as a good source of protein because of its high net protein value. We have obtained a net protein value of 30.1 for brewers' yeast, 21.2 for *Torula* yeast no. 1084 and 20.0 for *Torula* yeast no. 3. Even in the case of the food yeasts, with biological values below that of brewers' yeast, the net protein values were the same or above those of other foods such as raw soy flour 21.4% (Mitchell et al., '45), and rolled oats, navy beans, white flour and whole corn, which were 10.6, 6.0, 5.0 and 3.5%, respectively (Mitchell, '27). This proves that food yeast ranks high as a source of dietary protein.

SUMMARY

Using the nitrogen balance method, the protein values of 2 samples of food yeast *(Torula utilis)* and one of brewers' yeast were determined. The *Torula* yeasts, no. 1084 and no. 3, and the brewers' yeast were found, respectively, to be 88.3,

87.3 and 85.5% digestible, to possess biological values of 48.8, 45.3 and 69.3 and to have net protein values of 21.2, 20.0 and 30.1.

The 2 *Torula* yeasts, the one from England, no. 1084, and the one from Puerto Rico, no. 3, have practically equal nutritive coefficients. The superior net protein value of the brewers' yeast over that of the *Torula* samples is due exclusively to its higher biological value since the protein (N $\times$ 6.25) content and digestible coefficients of the 3 yeast samples are practically the same.

LITERATURE CITED

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1935 Official and Tentative Methods of Analysis. Fourth ed.: 25.

AXTMAYER, J. H. 1946 Oral communication to the College of Chemists of Puerto Rico., Oct. 13, 1946.

HUGHES, C. W., AND S. M. HAUGE 1945 Nutritive value of distillers' dried solubles as a source of protein. J. Nutrition, *30:* 245.

MITCHELL, H. H. 1924a A method of determining the biological value of protein. J. Biol. Chem., *58:* 873.

——— 1924b The biological value of proteins at different levels of intake. J. Biol. Chem., *58:* 905.

——— 1924c The supplementary relations among proteins. J. Biol. Chem., *58:* 923.

——— 1927 The protein values of foods in nutrition. J. Home Econ., *19:* 122.

MITCHELL, H. H., AND G. G. CARMAN 1924 The biological value for maintenance and growth of the proteins of whole wheat, eggs and pork. J. Biol. Chem., *60:* 613.

——— 1926 The biological value of the nitrogen of mixtures of patent white flour and animal foods. J. Biol. Chem., *68:* 183.

MITCHELL, H. H., T. S. HAMILTON AND J. R. BEADLES 1945 The importance of commercial processing for the protein value of food products. J. Nutrition, *29:* 13.

STILL, E. U., AND F. C. KOCH 1928 The biological value of yeast for the rat. Am. J. Physiol., *87:* 225.

THOMAS, K. 1909 Über die biologische Wertigkeit der Stickstoffsubstanzen in verschiedenen Nahrungsmittel. Arch. Anat. Physiol., vol. (1909), p. 219.

METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE

XI. VITAMIN C IN DIETS, BREAST MILK, BLOOD AND URINE OF NURSING MOTHERS [1]

BERTHA MUNKS, MILDRED KAUCHER, ELSIE Z. MOYER, MARY ELLEN HARRIS AND ICIE G. MACY

Research Laboratory, Children's Fund of Michigan, Detroit

(Received for publication January 30, 1947)

As part of a comprehensive investigation of the vitamin and mineral composition of human milk and the metabolism of women during the reproductive cycle we have determined nursing mothers' intakes of vitamin C, their secretion of the vitamin in milk, excretion in the urine, and the amounts in the cord blood as well as the levels in the blood of both mother and child. Other investigations have not included analyses of 24-hour collections of food, milk, and urine during the 10 days of the puerperium and at intervals during mature milk production, accompanied by determinations of vitamin C in the blood of the same subjects and their infants. The intake of vitamin C provided for the infant by his mother's milk will be discussed in another paper.

EXPERIMENTAL

The subjects of the investigation were multiparas with medical records of good or excellent health and of having successfully nursed their other children. Every effort was

[1] The investigation represented in part by this paper was partially supported by a grant from The Nutrition Foundation, Inc., and was made possible by the cooperation of Dr. J. P. Pratt, Chief of the Department of Obstetrics, Dr. B. M. Hamil, Department of Pediatrics, Elizabeth Moran, Director of Nurses, and Annie Lou Wertz, dietitian, all of the Henry Ford Hospital, Detroit.

made to standardize any procedure involving controllable factors. Vitamin C was determined by analysis of samples representing the total intake of vitamin-C-containing foods for 24-hour periods, thus obtaining quantitative estimations of the amounts ingested. Qualitatively the diets were similar for all women during all 5-day periods (Kaucher et al., '45, '46); however there were differences in consumption owing to variations in appetites and food composition. Analyses of complete 42-hour collections of milk and urine obtained during the first 10 days postpartum and during each day of 5-day periods at intervals during lactation avoided portrayal of diurnal changes in concentration in the results. In addition, vitamin C was determined in placental tissue (Pratt et al., '46), in blood from the umbilical cord and in capillary blood obtained from both mother and infant on the first and tenth days after delivery, and the first and last days of 5-day periods of study. To obtain comparable data, the blood samples were taken from mother and child in the morning after expression of the milk but before breakfast.

Analyses of food, milk and urine were made by the method of Roe and Kuether ('43); of blood, by the method of Farmer and Abt ('36). Preceding publications have given details of selection of subjects (Macy, Williams, Pratt and Hamil, '45), diet and procedures of sampling and preparation of food composites and milk (Kaucher et al., '45), the method of manually expressing milk (Davies, '45) and collection and analysis of urine (Roderuck, Williams and Macy, '46). The concentration of vitamin C in immature and mature human milk has been presented (Munks et al., '45).

RESULTS

The volumes of milk secreted and urine excreted by 4 women during each of the first 10 days postpartum, the intakes of vitamin C per day and the amounts of vitamin C in the immature milk and in the urine for each day are given in table 1.

Within each 5-day period there were wide variations from day to day in vitamin C intake, depending upon the kinds of

TABLE 1

Vitamin C intake, secretion and excretion during first 10 days postpartum.

SUBJECT	INTERVAL POSTPARTUM	VOLUME		VITAMIN C		
		Milk	Urine	Intake	Milk	Urine
	days	*ml*	*ml*	*mg*	*mg*	*mg*
V.K.	1	9	3086	148		24.9
	2	90	3651	112		15.7
	3	484	2596	77	50	13.4
	4	547	2427	128	54	11.6
	5	560	2331	83	50	12.4
	6	663	2249	126	60	10.3
	7	781	3380	115	61	8.3
	8	775	3840	63	70	9.6
	9	794	3115	176	70	9.8
	10	797	3328	51	61	3.5
V.L.	1	30	1757	138	1	23.9
	2	56	2686	98	4	14.9
	3	353	2759	86	25	11.4
	4	794	1823	204	50	13.4
	5	844	1847	98	55	14.1
	6	955	2710	200	63	18.2
	7	1047	2841	161	69	14.2
	8	1098	2066	87	76	14.2
	9	1118	2151	183	73	11.8
	10	1200	1669	99	83	9.2
J.M.	1	35	2182	216	2	8.9
	2	385	1386	147	23	6.4
	3	870	1519	96	44	8.2
	4	1011	1201	201	56	6.5
	5	1121	1283	114	62	8.5
	6	1125	1172	169	51	4.7
	7	1287	1161	151	75	6 4
	8	1136	1520	92	55	6.6
	9	1258		254	83	
	10	1336	1553	59	87	7.5
V.S.	1	6	1869	204		34.7
	2	92	1874	98	8	24.9
	3	420	2887	98	37	28.2
	4	600	2063	207	49	35.4
	5	697	2065	112	54	26.4
	6	756	1502	156	54	22.8
	7	818	2524	154	65	22.0
	8	837	2237	98	67	20.9
	9	932	1984	196	76	19.2
	10	924	2287	89	65	15.0

foods included in the diet for the different days. The lowest vitamin C intake for any day was 51 mg, the highest was 254 mg. The average daily consumption for the 10 days was 134 mg, an amount lower than the 150 mg recommended by the Food and Nutrition Board of the National Research Council ('45). However, it is important in evaluating the data on vitamin C to remember that the values represent intakes determined by analysis of food *as eaten*, by 1 method of determination, whereas the Recommended Allowances are based upon data obtained by various analytical methods and were planned to include ample amounts as "safety factors."

It is evident that the amounts of vitamin C secreted in the milk each day were not related to the daily fluctuations in intake, but paralleled the increased production of milk, secondarily influenced by the slightly augmented ascorbic acid concentration (Munks et al., '45). The high excretions of vitamin C in the urine by 3 of the mothers after delivery, followed by decreases of 50% or more within the first 10 days postpartum, parallel the decreased urinary excretions of vitamin C reported for newborn infants (Hamil et al., '47) and support the belief that the tissues of both mother and fetus require greater concentrations of vitamin C for saturation during pregnancy than during lactation.

Subject V.S., who had been taking 100 mg of ascorbic acid per day throughout pregnancy, in addition to an excellent diet and supplements of other vitamins (Roderuck, Williams and Macy, '46; Roderuck, Coryell, Williams and Macy, '46) secreted milk with the highest concentration of vitamin C each day of the puerperium and also excreted greater amounts in urine (15.0 to 35.4 mg) than did any of the other women. Subject J.M., whose milk flow was established more rapidly than that of the other 3 women and reached the highest level during the first 10 days, excreted much less vitamin C in urine during the first few days after delivery, but continued to excrete at approximately the same level regardless of the increased secretion in milk.

Despite the similarity of the women's average daily intakes during each 5-day period and the relationship between volume of milk produced and its vitamin C content, the data in table 1 do not indicate a relationship between volume of milk or its vitamin C content and excretion of the vitamin in urine. Nor was urine volume a factor in excretion of vitamin C, which ranged from 3.5 mg in 3328 ml to 35 mg in 1869 ml of urine. Several possibilities are suggested by the urine values. If the level of tissue saturation required during lactation is lower than that demanded by pregnancy, the decline of over 50% in the daily excretion of vitamin C by 3 women during the first 10 days postpartum may represent an "unloading" from the maternal tissues. If this is true it would indicate that the tissues of subject J.M. had not been saturated during the prepartum period or that the amount which she might have "unloaded" after delivery was expended during labor and childbirth. That the woman's prepartum nutritional status was not poor is evidenced by the medical records, by her quick establishment of lactation, and the secretion in milk of greater amounts of vitamin C per day than any other woman. The fact that J.M. maintained a level of excretion in urine ranging from 5 to 9 mg of vitamin C per day also indicates that the decreased excretions by the other women are not attributable to the demands of increased milk production. The possibility that J.M. was excreting required minimal amounts in urine is doubtful because the urinary vitamin C of V.K. and V.L. declined to comparable levels by the tenth day postpartum.

The vitamin C contents of the serum of the cord blood and the blood of mother and infant, shown in table 2, contribute additional information on the utilization of the vitamin. Except for subject L.F., the cord blood of all women had a greater concentration of vitamin C than did blood taken from mothers and infants on the first day postpartum. In all but one instance (J.M.) blood vitamin C levels for both mother and child were lower on the tenth than on the first day postpartum, and the levels in the infant's blood were as high or

higher than those in their mothers' blood. The cord blood values ranged from 0.4 to 2.2 mg vitamin C per 100 ml. The infant blood contained 0.2 to 2.2 mg and the maternal blood from 0.2 to 1.4 mg per 100 ml during the first 24 hours postpartum. These values are within the range given by other workers. On the tenth day postpartum the infant and maternal vitamin C blood serum concentration ranged from 0.4

TABLE 2

Blood serum vitamin C during the first 10 days postpartum. (mg/100 ml.)

SUBJECT	CORD	INTERVAL POSTPARTUM	MOTHER	CHILD
		days		
V.K.	1.3	1	0.6	1.0
		10	0.4	0.8
V.L.	2.2	1	0.6	0.7
		10	0.4	0.4
J.M.	0.4 [1]	1	0.2	0.2
		10	0.1	0.5
V.S.	1.5	1	0.3	1.0
		10	0.3	0.6
L.F.	1.6	1	1.4	2.2
		10	0.8	1.9
V.G.		1	1.3	2.2
		10	0.6	1.2
C.O.	1.5	1	0.7	
		10	0.1	0.4

[1] Placental.

to 1.9 mg and from 0.1 to 0.8 mg, respectively. The values for cord, mothers' and infants' blood were within the ranges, respectively, found for 24 other healthy mothers and their infants during the first week of life (Hamil et al., '47).

The values in tables 1 and 2, supported by the values presented earlier (Hamil et al., '47) for vitamin C in cord and maternal blood during the first week postpartum, emphasize the inconclusiveness of determinations of vitamin C levels in either blood or urine, or both, as an index of nutritional status with respect to vitamin C during lactation. Among women receiving diets undoubtedly superior to those of most mothers

during the puerperium, with appetite-inhibiting factors eliminated (coffee, tea, candy, etc.), vitamin C in the maternal blood and urine may be at levels generally considered to indicate scurvy (J.M.), yet not interfere with secretion of large volumes of milk containing ample amounts of the vitamin. Levels of vitamin C in the blood may also be low in subjects known to have been "saturated" before delivery (V.S.) although large amounts are being excreted in the urine. Recent work indicates that the ascorbic acid content of the white blood cells parallels the retention and may be a reliable index of total body concentration of ascorbic acid (Lowry et al., '47) in normal adults. Whether similar results would be obtained with nursing mothers is not known.

The variations in the vitamin C contents of milk, blood, and urine from healthy mothers receiving excellent diets containing 51 to 254 mg of vitamin C per day, determined under conditions as closely controlled as possible, indicate a wide range of normal, individual physiologic differences. Data obtained under the conditions of the study are not comparable to those procured with subjects receiving low intakes of vitamin C from inferior diets or high intakes from supplemental sources; nor can they be compared closely with results obtained with non-lactating women or those obtained with dietary intakes calculated from tables of food values. Further research is needed to clarify the influences exerted by the many factors involved in the vitamin C metabolism of pregnant and nursing women. Among these are the possibility of fetal synthesis of the vitamin, physiologic adjustment of the maternal body from conditions of pregnancy to those of lactation, including possible reorganization of the glands of internal secretion, the level of requirement for the vitamins as opposed to maximum level of storage (saturation), and differences in the requirements before pregnancy, during gestation, and the lactation period.

The intakes of vitamin C by 6 women during 13 5-day periods in which they were secreting mature milk, are given in table 3, with the volumes of milk secreted and urine excreted

TABLE 3

Daily vitamin C intakes, secretion in mature milk and excretion in urine.

SUBJECT	INTERVAL POSTPARTUM	VOLUME		VITAMIN C		
				Daily total		
		Milk	Urine	Intake	Milk	Urine
	days	*ml*	*ml*	*mg*	*mg*	*mg*
V.G.	78	683	1663	178	36	4.8
	79	846	1359	112	44	4.3
	80	923	1419	70	51	5.4
	81	874	844	194	52	6.5
	82	913	1120	86	49	6.2
	161	759	989	94	39	5.4
	162	848	801	118	43	7.3
	163	912	813	75	46	5.8
	164	985	1005	130	46	8.2
	165	1000	1359	108	51	2.0
	239	610	853	136	48	61.2
	240	624	731	112	48	34.5
	241	661	736	84	50	43.2
	242	738	958	104	41	47.5
	243	772	770	72	54	33.4
	302	414	1350	189		39.2
	303	389	1068	140	30	33.4
	304	383	949	120	30	30.3
	305	389	1099	171	30	42.0
	306	394	933	96	27	23.0
V.K.	95	544	3072	115	36	10.6
	96	615	3443	106	39	11.2
	97	701	2587	91	45	6.9
	98	691	3206	139	44	10.4
	99	684	1710	61	44	8.3
	144	272	2739	132	22	12.8
	145	315	1286	136	26	11.5
	146	343	1476	180	29	13.3
	147	323	827	155	25	13.5
	148	372	907	121	28	10.6
V.L.	68	736	3408	180	49	10.4
	69	743	4154	140	46	11.8
	70	802	2838	195	53	12.8
	71	833	1903	175	49	6.8
	72	829	1940	120	51	10.5

TABLE 3 (*continued*)

SUBJECT	INTERVAL POSTPARTUM	VOLUME		VITAMIN C		
				Daily total		
		Milk	Urine	Intake	Milk	Urine
	days	*ml*	*ml*	*mg*	*mg*	*mg*
V.L.	152	684	2457	227	47	68.9
	153	671	1970	145	43	26.9
	154	673	1637	105	43	23.9
	155	686	1484	218	44	46.8
	156	686	1232	85	39	17.2
J.M.	75	598	2517	153	27	6.3
	76	677	2081	110	29	6.4
	77	740	2155	171	29	0.0
	78	742	2646	157	34	7.6
	79	783	1925	100	38	4.0
	173	205	2993	183	12	5.0
	174	231	1922	156	13	3.6
	175	296	2089	79	16	4.0
	176	301	1169	129	16	4.2
	177	309	1841	108	15	5.1
B.S.	204	864	1630	158	32	6.0
	205	927	1350	133	35	7.2
	206	918	1231	79	37	6.3
	207	938	1219	200	41	6.2
	208	918	1352	78	40	5.9
	259	734	1665	146	37	7.6
	260	612	1299	95	29	7.0
	261	631	1037	76	30	7.7
	262	725	979	166	34	4.8
	263	678	1113	89	36	7.8
V.S.	70	234	1897	315	23	141.3
	71	276	1846	202	29	78.4
	72	334	2308	200	31	75.1
	73	315	1938	275	30	118.3
	74	361	1269	148	32	48.7

per 24 hours and their contents of the vitamin. During 10 of the periods the mothers' volumes of milk were increased 54 to 241 ml per day as the result of regular and complete emptying of the breasts by manual expression (Macy, Hunscher, Donelson and Nims, '30). Also for some of the women there

was an improvement of diet during the experimental periods. Although the diets for all 5-day periods were comparable, qualitatively, and appetites were satisfied by proportionate adjustment of all foods except milk, the vitamin C intakes as determined by analysis varied greatly owing to differences in concentration of the vitamin in the same foods. For example, V.L., J.M., and V.S. received diets of equal amounts, respectively, of the same foods (table 3). Calculated from the literature their average daily intakes of vitamin C during the 5-day periods ranged from 166 to 169 mg. The analyses for the different intervals of study averaged from 131 to 228 mg per day.

There were large differences in the vitamin C intakes for the 5 days of each study period owing to inclusion of foods with high contents of the vitamin in some of the menus. However, the concentration of vitamin C in mature milk was quite constant, averaging 5 mg per 100 ml (Munks et al., '45), and in general the daily secretion of the vitamin paralleled changes in milk volume. Although for the well-nourished mothers no direct relationship was found between the variable vitamin C intakes and the amount secreted in the 24-hour collections of milk, other work has shown vitamin C secretion to be generally dependent on diet (King, '38; Smith, '38; Escudero and Pierangeli, '43). Escurdero and Pierangeli have reported that with an intake of approximately 70 mg vitamin C per day from food during the last month of pregnancy and the colostral period, the colostrum averaged 2.2 mg % vitamin C. Women receiving 240 mg produced colostrum containing 5.5 mg per 100 ml. Selleg and King ('36) found that the relatively slow and limited rise in C value when patients received a special orange juice supplement provides an indication of a maximum and approximately optimum level of secretion, above which an excessive dietary intake results chiefly in a rapid urinary excretion without disturbing the lactation level. Such results emphasize the ample provision of vitamin C by the diets eaten by the nursing mothers in this study.

Levels of excretion in the 24-hour collection of urine were not related to differences in level of intake among the women of this study, nor to the vitamin C secretion in milk, again indicating that individual characteristics conditioned by prior nutritional status with respect to the vitamin were the major controlling factors. It has been shown that in addition to tissue saturation excretion of vitamin C in urine may be influenced by the individual's kidney threshold for the vitamin (Faulkner and Taylor, '38), by the fluid intake, and by environmental temperature and humidity (Shields and coworkers, '46) all of which were beyond the control of the present study. It is likely that other individual characteristics are involved, so it is not surprising to find wide variations in the urinary vitamin C among individuals and for the same individual at different times during lactation.

The concentrations of vitamin C in the blood serum of both the mothers and infants during the 5-day periods of mature milk production are given in table 4. The average values correspond for all determinations on mothers' blood during mature milk secretion (0.45 mg) and during the first 10 days postpartum (0.56), and the distributions are similar. The average of the values for the breast-fed infants when 2 to 10 months old (1.1 mg) approximates that for the first 10 days postpartum (1.0 mg). Fourteen of the samples from the mothers were obtained on the first days of study periods; 5 were obtained after the women had received the study diet for 5 days. The values for all 5 were the same or higher than those of the first days of study, respectively, attesting the adequacy of the study diet and perhaps indicating its superiority over their usual food intakes. However, this conclusion is precluded by the secretions in milk, the levels in the infants' blood, and by the excretions in the urine, especially those of V.S. who had been ingesting 100 mg of ascorbic acid per day with a good diet, prior to the study, who secreted milk with the highest concentration of the vitamin (10 mg per 100 ml), yet whose blood level on the first day (0.1 mg) was lower than any other value obtained and increased during the 5 days with

an intake undoubtedly lower than that of her previous supplemented diet.

It is evident that milk secretion requires that the requisite substances be supplied through the blood, either from the products of digestion or from the tissues. The best estimates

TABLE 4

Blood serum vitamin C during mature milk secretion. (mg/100 ml.)

SUBJECT	INTERVAL POSTPARTUM	MOTHER	CHILD
	days		
V.G.	78	0.3	0.5
	161	0.2	1.7
	239	0.6	0.8
	244 [1]	1.0	
	302	0.2	1.1
	307 [1]	0.6	
V.K.	95	0.8	3.0
	144	0.7	0.9
V.L.	68	0.3	1.5
	152	0.8	1.4
	157 [1]	0.8	
J.M.	75	0.2	0.7
	172	0.2	0 6
	177 [1]	0.6	
B.S.	85	0.2	1.0
	204	0.2	0.8
	259	0.4	0.6
V.S.	70	0.1	0.8
	75 [1]	0.4	

[1] After receiving study diet for 5 days.

are that 400 to 500 volumes of blood are required to produce 1 volume of milk (Kay, '45). Obviously, withdrawal from the blood parallels the rate of secretion, which is determined by the fullness of the gland. It is likely that, for vitamin C at least, the blood level is more easily replenished from the supply provided by food than by mobilization from body stores.

Whether the level of the vitamin in the blood of nursing mothers changes rapidly in response to emptying of the breasts and also varies in relation to the intervals between meals is being investigated.

The net gain or loss of vitamin C to the body, estimated by subtraction of the values for urine and milk from the intakes, indicates the amount of the vitamin used in metabolism and stored in the tissues, or the deficit which has been withdrawn from the tissues. The quantity of vitamin C excreted through the skin would seem inappreciable, since recent work has shown that at comfortable temperatures the loss may amount to 0.8 mg daily and even under conditions of induced profuse sweating the quantity is only 2.7 mg per day (Shields, et al., '46). In the 10-day puerperium the total quantities of vitamin C consumed by V.K., V.L., J.M., and V.S. were 1079, 1354, 1499 and 1412 mg, respectively (table 1). During the same period the women secreted into their milk, totals of 476, 499, 538 and 475 mg of vitamin C. Their urine contained 120, 145, 64 and 250 mg, leaving for metabolic function and tissue storage 483, 710, 897 and 687 mg, respectively. There were wide individual variations among these 4 women as shown by the range of 113 to 205 mg per day which was unaccounted for immediately following recovery from the trauma of labor and delivery, about twice the 25 to 50 mg daily observed in persons in a state of saturation (King, '38). It is conceivable that during this recuperation period there are appreciable changes in the acid-base equilibrium of the body. This is supported by evidence that the quantity of vitamin excreted in the urine may be varied merely by changing the acid-base balance of the food intakes (Hawley and co-workers, '36).

Twenty-seven of 64 daily determinations with women secreting mature milk showed more than 90 mg per day unaccounted for on days when the intakes were high (table 3), which indicates, perhaps, that the mothers were not saturated and the amount was dependent on the intake. The greatest amount unaccounted for was 166 mg per day, opposed to a loss of 15.4 mg per day from the mother's tissue stores. During

a 5-day study in the eighth month postpartum, V.G. had an average intake of 101.6 mg vitamin C and a secretion of 48.2 mg in the milk. These values are similar to those of the same woman when studied at 3 and 6 months, yet the excretion of vitamin C in the urine was 44 mg per day, over 8 times the average excretion during the earlier studies and leaving only 9.4 mg vitamin C per day unaccounted for. The blood serum vitamin C concentration was 0.6% on the first day of the study and increased to 1.0 mg % on the fifth day of the study (table 4). If the daily body requirement for vitamin C is 50 to 100 mg per day or if the mother's indispensable minimum requirement was 25 mg per day (King, '38) it is difficult to understand why she excreted an average of 44 mg vitamin C per day through the kidneys, unless she previously had a high vitamin C intake which continued to be excreted even after the intake was lowered. This phenomenon has been demonstrated by Harris, Ray and Ward ('33), Harris and Ray ('35) and Hess and Benjamin ('34), who found the urinary output to depend on both the immediate vitamin C intake and also on the post-nutritional history or state of "saturation."

When studied during the tenth month of lactation the same woman's average intake was 143.2 mg per day. The vitamin C excreted in the milk had dropped almost one-half, 29.2 mg per day, owing to decreased volume. Her urine excretion, 33.6 mg per day, still was higher than during the third and sixth months. The vitamin C unaccounted for had returned to the former range of 80 mg average per day. The need for this increased retention was shown by the blood serum concentration which was only 0.2 mg per 100 ml at the beginning of the study but increased to 0.6 by the fifth day (table 4).

A similar saturation was demonstrated by V.L. during her third and sixth months postpartum. The average intakes of vitamin C were 162 and 156 mg, secretion in the milk amounted to 49.6 and 43.2 mg, and the urine excretion averaged 10.5 and 36.7 mg of vitamin C per day, respectively. At the beginning of the study at 3 months the blood serum level was 0.3 mg

per 100 ml but at the beginning of the 5-day study at 6 months, when she was throwing off greater amounts of vitamin C in urine, her blood level was 0.8 mg of vitamin C per 100 ml. Large vitamin C excretions may occur even when the blood serum vitamin C is low, if the dietary intake is high. For example, V.S. in the third month of lactation excreted 92.4 mg of vitamin C per day, yet she had a blood serum level of only 0.1 mg % vitamin C on the first day of the study. Her average dietary intake was 228.0 mg vitamin C over the 5-day period and was the highest average intake during any 5-day study.

The amount of vitamin C which appears in the urine is also affected by the way in which the daily intake is distributed within the 24 hours. Widenbauer and Kühner ('37) and Ralli, Friedman and Sherry ('39) have emphasized the importance of dividing the intake into small doses throughout the day. Todhunter and Robbins ('40) have estimated the minimum daily intake of vitamin C required to maintain the tissues in a state of complete saturation to be 1.6 to 1.7 mg per kg of body weight per day for college women. Ralli, Friedman and Sherry ('39) concluded from their experiments that 100 mg per day be suggested as the requirement of women for saturation. With the exception of a woman who weighed 73.5 kg, the daily requirement of the lactating women in this study would have been 100 mg per day or less by this criterion. If one adds the amounts of vitamin C secreted in milk to the amounts calculated from their weights to be the mother's requirements, one obtains an estimated daily requirement for lactating women ranging from 106.8 to 174.1 mg of vitamin C per day. During the first 10 days postpartum the vitamin C intakes of 2 women met the estimated dietary requirement. For the woman who weighed 73.5 kg the estimated requirement was greater than the average daily intake during the first 10 days postpartum. During the third month of lactation the diet supplied this theoretical requirement for saturation in 3 of 5 women.

One of the lowest blood values, 0.1 mg vitamin C per 100 ml, was recorded for a woman who secreted the highest concentra-

tion of vitamin C in milk, 10 mg per 100 ml. Other women having blood serum values up to 0.8 mg per 100 ml did not secrete as great concentrations of vitamin C in the milk. One, V.G., was known to secrete over 7 mg per 100 ml in her milk when her blood serum vitamin C ranged anywhere from 0.2 to 1.3 mg per 100 ml. These data confirm the observation of Ingalls, Draper and Teel ('38) that there is no direct correlation between the blood serum vitamin C and the concentration in the milk. However, they state that "the fact that mothers on a diet relatively low in vitamin C will secrete in the milk amounts of ascorbic acid so large that the ascorbic acid content of the infant's plasma is higher than that of the mother's is indicative of an interesting biologic mechanism whereby the child receives the vitamin at the expense of its mother." For the lactating mother and the breast-fed infant vitamin C levels in the blood, as well as in urine, may be closely correlated with eating time. In this study blood samples were taken following collection of milk and urine at 6:00 A. M. It is possible that the low values for the mother's blood were the result of the demands for milk production during the preceding 12 hours of fasting. If this is correct then the supply in the blood would be replenished immediately after eating breakfast and the low fasting values would not indicate that the child receives vitamin C "at the expense of the mother."

Until requirements can be more clearly distinguished from "storage" or "saturation," it is obvious that for the physician nutrition during the nursing period is a problem similar to that stated for pregnancy by Lund ('45): ". . . the problem is the dietary needs of each patient. She is not to be treated according to the nutritional standards of any other person or groups of persons. She is an individual problem and it is the physician's duty to take a short, accurate dietary history and to see that he or some other qualified person explains to her the dietary problems of pregnancy." From the present study it seems that the Recommended Dietary Allowances ('45) of the Food and Nutrition Board of the National Research Council provide for adequate amounts of

vitamin C to meet normal requirements during lactation and prevent depletion of body stores, provided during pregnancy the nutritional status of the mother has been adequate with respect to the vitamin.

SUMMARY

Vitamin C was determined by analysis in the food as eaten each day by healthy nursing mothers and in 24-hour collections of their milk and urine during the first 10 days postpartum and 5-day periods at intervals later in lactation. In addition, vitamin C was determined in blood from the umbilical cord and in fasting samples of capillary blood from the mothers and infants.

The amounts of vitamin C secreted in milk paralleled the increases in milk volume, while vitamin C excretion in the urine was high after delivery and for 3 or 4 mothers decreased 50% or more by the tenth day. No relationships between volume of milk or its vitamin C content and excretion in the urine were indicated.

During the periods of secretion of mature milk, the amounts of vitamin C in the milk, in general, paralleled milk volume. For the mothers studied, whose nutritional status had been good during pregnancy, no relationship was found between vitamin C intake and secretion in milk. Excretion in urine was not related to level of intake or secretion in milk.

Extreme anatomic and physiologic variability among individuals and in the same individual at different times is emphasized by the results. The data indicate also the inconclusiveness of determinations of vitamin C levels in milk, urine or fasting blood samples as an index of vitamin C nutritional status of lactating women receiving good diets. The need for further research on the physiologic influences exerted by lactation and pregnancy on vitamin C metabolism is stressed. These include adjustment of the maternal body from pregnancy to lactation, including the glands of internal secretion, the level of requirement as opposed to maximum level of storage, and differences in requirements before pregnancy,

during gestation, and during lactation. On the basis of the data presented it seems that the Recommended Dietary Allowances provide for adequate amounts of vitamin C to meet normal requirements during lactation, provided the prenatal nutritional status of the mother has been satisfactory.

LITERATURE CITED

DAVIES, V. 1945 Human milk studies XXI. A simple technic for the manual expression of mother's milk. Am. J. Dis. Child., *70:* 148.

ESCUDERO, P., AND E. PIERANGELI 1943 Influencia de la alimentacion de la gravida sobre la riqueza mineral y vitaminica del calostro. Revista de la Asociacion Argentina de Dietologia, *1:* 85.

FARMER, C. J., AND A. F. ABT 1936 Determination of reduced ascorbic acid in small amounts of blood. Proc. Soc. Exp. Biol. Med., *34:* 146.

FAULKNER, J. M., AND F. H. L. TAYLOR 1938 Observations on the renal threshold for ascorbic acid in man. J. Clin. Invest., *17:* 69.

FOOD AND NUTRITION BOARD 1945 Recommended Dietary Allowances. National Research Council, Reprint and Circular Series 122.

HAMIL, B. M., B. MUNKS, E. Z. MOYER, M. KAUCHER AND H. H. WILLIAMS 1947 Vitamin C in the blood and urine of the newborn during the first week of life and in the cord and maternal blood. Am. J. Dis. Child. (in press.)

HARRIS, L. J., AND S. N. RAY 1935 Diagnosis of vitamin-C subnutrition by urine analysis, with a note on the antiscorbutic value of human milk. Lancet, *1:* 71.

HARRIS, L. J., S. N. RAY AND A. WARD 1933 The excretion of vitamin C in human urine and its dependence on the dietary intake. Biochem. J., *27:* 2011.

HAWLEY, E. E., J. B. FRAZER, L. L. BUTTON AND D. J. STEPHENS 1936 The effect of the administration of sodium bicarbonate and of ammonium chloride on the amount of ascorbic acid found in the urine. J. Nutrition, *12:* 215.

HESS, A. F., AND H. R. BENJAMIN 1934 Urinary excretion of vitamin C. Proc. Soc. Exp. Biol. Med., *31:* 855.

INGALLS, T. H., R. DRAPER AND H. M. TEEL 1938 Vitamin C in human pregnancy and lactation. II. Studies during lactation. Am. J. Dis. Child., *56:* 1011.

KAUCHER, M., E. Z. MOYER, A. J. RICHARDS, H. H. WILLIAMS, A. L. WERTZ AND I. G. MACY 1945 Human milk studies. XX. The diet of lactating women and the collection and preparation of food and human milk for analysis. Am. J. Dis. Child., *70:* 142.

KAUCHER, M., E. Z. MOYER, H. H. WILLIAMS AND I. G. MACY 1946 Adequacy of the diet during lactation. J. Am. Diet. Assoc., *22:* 594.

KAY, H. D. 1945 The secretion of milk. Nature, *156:* 159.

KING, C. G. 1938 The physiology of vitamin C. J. Am. Med. Assoc., *111:* 1098.

LOWRY, O. H., O. A. BESSEY, M. J. BROCK AND J. A. LOPEZ 1947 The interrelationship of dietary, serum, white blood cell, and total body ascorbic acid. J. Biol. Chem., *166:* 111.

LUND, C. J. 1945 Nutrition in pregnancy. J. Am. Med. Assoc., *128:* 344.

MACY, I. G., H. A. HUNSCHER, E. DONELSON AND B. NIMS 1930 Human milk flow. Am. J. Dis. Child., *39:* 1186.

MACY, I. G., H. H. WILLIAMS, J. P. PRATT AND B. M. HAMIL 1945 Human milk studies. XIX. Implications of breast feeding and their investigation. Am. J. Dis. Child., *70:* 135.

MUNKS, B., A. ROBINSON, H. H. WILLIAMS AND I. G. MACY 1945 Human milk studies XXV. Ascorbic acid and dehydroascorbic acid in colostrum and mature milk. Am. J. Dis. Child., *70:* 176.

PRATT, J. P., C. RODERUCK, M. CORYELL AND I. G. MACY 1946 Composition of the human placenta. III. Vitamin content. Am. J. Obstet. Gynec., *52:* 783.

RALLI, E. P., G. J. FRIEDMAN AND S. SHERRY 1939 The vitamin C requirement of man, estimated after prolonged studies of the plasma concentration and daily excretion of vitamin C in 3 adults on controlled diets. J. Clin. Invest., *18:* 705.

RODERUCK, C., M. CORYELL, H. H. WILLIAMS AND I. G. MACY 1946 Metabolism of women during the reproductive cycle. IX. The utilization of riboflavin during lactation. J. Nutrition, *32:* 267.

RODERUCK, C., H. H. WILLIAMS AND I. G. MACY 1946 Metabolism of women during the reproductive cycle. VIII. The utilization of thiamine during lactation. J. Nutrition, *32:* 249.

ROE, J. H., AND C. A. KUETHER 1943 The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J. Biol. Chem., *147:* 399.

SELLEG, I., AND C. G. KING 1936 The vitamin C content of human milk and its variation with diet. J. Nutrition, *11:* 599.

SHIELDS, J. B., B. C. JOHNSON, T. S. HAMILTON AND H. H. MITCHELL 1946 The excretion of ascorbic acid and dehydroascorbic acid in sweat and urine under different environmental conditions. J. Biol. Chem., *161:* 351.

SMITH, S. L. 1938 Human requirements of vitamin C. J. Am. Med. Assoc., *111:* 1753.

TODHUNTER, E. N., AND R. C. ROBBINS 1940 Observations on the amount of ascorbic acid required to maintain tissue saturation in adults. J. Nutrition, *19:* 263.

WIDENBAUER, F., AND A. KÜHNER 1937 Ascorbinsäurestudien an stillenden Frauen. Zeitschr. f. Vitaminforsch., *6:* 50.

METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE

XII. THE EFFECT OF MULTI-VITAMIN SUPPLEMENTS ON THE VITAMIN C UTILIZATION OF NURSING MOTHERS [1]

J. P. PRATT, BERTHA MUNKS AND ICIE G. MACY
The Henry Ford Hospital; and the Research Laboratory, Children's Fund of Michigan, Detroit

(Received for publication January 30, 1947)

A preceding paper (Munks, Kaucher, Moyer, Harris and Macy, '47) reported analyses for vitamin C in the food intakes, milk, urine and blood of healthy mothers, and in the blood of their infants at various periods during lactation. The results demonstrated the wide variations in vitamin C metabolism among normal lactating women and the unreliability of levels of the vitamin in the blood and urine as an index of nutritional status with respect to the vitamin. Intakes comparable with the Recommended Allowances ('45) of the Food and Nutrition Board, from good diets of natural foods, were shown to be ample in meeting the health requirements of mothers while they were nursing their infants, although urine excretion of vitamin C was extremely variable and fasting blood levels were frequently as low as those considered to indicate scurvy in non-pregnant, non-lactating women. To extend the information concerning vitamin C metabolism in nursing mothers, the milk, urine and blood of the same subjects and the blood of their infants were analyzed after 6 to 14 days during which the women received supplemental amounts of ascorbic acid and other synthetic vitamins in

[1] The investigation represented in part by this paper was partially supported by a grant from the Nutrition Foundation, Inc.

addition to good diets. These data may be compared with the average values for the same subjects during 5-day periods of known intake immediately preceding the interval of supplement (Munks, et al., '47).

PROCEDURE

During 13 periods of 5 consecutive days for 6 healthy mothers who were successfully nursing their infants, vitamin C was determined in 24-hour collections of food, as eaten, urine and milk. Blood levels of vitamin C were determined for both the mothers and their infants. For 6 to 14 days immediately following the 5-day periods the mothers received 750 mg of ascorbic acid, 50,000 U.S.P. units of vitamin A, 12 mg of thiamine chloride, 15 mg of riboflavin and 120 mg of niacin.[2] To distribute the intake of the vitamins over the day (Ralli, Friedman and Sherry, '39) the supplement of the water-soluble vitamins was given in 3 equal capsules, 1 with each meal. For the last day of the supplemented diet all milk and urine was collected and a fasting blood sample from the mother, after expression of the milk at 6 A.M., and a blood sample from the infant were obtained at the end of the 24 hours.

Analyses of food, milk and urine for vitamin C were made by the Roe and Keuther method ('43) and of the blood by the method outlined by Farmer and Abt ('36). Details of the selection of subjects (Macy et al., '45), diets (Kaucher, Moyer, Williams and Macy, '46), the methods of manually expressing the milk (Davies, '45) and procedures of collecting the food composites, milk and urine (Kaucher, Moyer, Richards, Williams, Wertz and Macy, '45) are given elsewhere. The concentration of vitamin C in colostrum and mature breast milk (Munks et al., '45) and the nutritional state of the nursing mothers with respect to vitamin C (Munks and coworkers, '47) have been recorded.

[2] The amounts of the vitamins given as supplements were less than therapeutic dosages, defined as 10 times the daily requirements, respectively.

RESULTS

Table 1 presents the average daily values for vitamin C in the food, breast milk and urine during the 5-day control periods and the results of analyses of 24-hour samples collected on the last day of supplementation of the diet with 750 mg of ascorbic acid.

The concentration of vitamin C in the milk was in some instances more than doubled and was lowered only for 1 study (V.G.) although several of the daily values were less after supplement, owing to the smaller volumes of milk secreted. However, the average concentration was increased only 2 mg, from 6 to 8 mg per 100 ml. The largest amount of vitamin C secreted per day after supplementation with 750 mg per day was 76 mg by V.G. during her third month of lactation. During the control studies the mothers, weighing from 55 to 73 kg, were eating daily diets which by analysis contained averages of 102 to 228 mg vitamin C, although by calculation from tables of food composition the vitamin C contents of the diets ranged from 132 to 169 mg per day.

Although there may be an upper limit to the concentration of vitamin C an individual woman can secrete in her milk, the limit does not seem to be the same for every individual. The highest value, 12.4 mg per 100 ml, was found in the milk of a woman (V.S.) in whom lactation was almost terminated. However, before the supplement was taken this woman was secreting milk containing 9.5 mg per 100 ml. Another woman secreted 10.1 and 10.5 mg per 100 ml, respectively, after supplementation in the third and sixth months of lactation, although during the control period her milk contained only 4.4 and 5.3 mg per 100 ml, respectively. After 4 supplementation periods at least 2 months apart, values for a third woman were changed from 5.4, 5.0, 7.0 and 7.5 mg to 7.5, 7.1, 3.8 and 7.5 mg per 100 ml, respectively.

The concentrations in urine following the supplement were as much as 140 times (V.G.) those of the control period. The total daily excretions after supplement ranged from 504 to 680 mg greater than the averages for the 5 days of the control

TABLE 1

Vitamin C in milk and urine before and after supplement.[1]

SUBJECT	CONTROL						AFTER SUPPLEMENT				
	Interval postpartum	Intake	Milk		Urine		Interval postpartum	Milk		Urine	
	days	*mg*	*mg/100 ml*	*mg/day*	*mg/100 ml*	*mg/day*	*days*	*mg/100 ml*	*mg/day*	*mg/100 ml*	*mg/day*
V.G.	78–82	128	5.4	46	0.4	5	92	7.5	76	56	531
	161–165	105	5.0	45	0.6	6	175	7.1	62		
	239–243	102	7.0	48	5.6	44	253	3.8	27	75	689
	302–306	143	7.5	29	3.2	34	316	7.5	24	66	632
V.K.	95–99	102	6.5	42	0.3	9	109	8.9	62	33	613
	144–148	145	8.0	26	0.8	12	158	9.8	32	60	516
V.L.	68–72	162	6.3	50	0.4	10	82	8.2	64	38	665
	152–156	156	6.4	43	2.2	37	170	6.8	35	47	717
J.M.	75–79	138	4.4	31	0.2	5	89	10.1	61	18	612
	173–177	131	5.3	14	0.2	4	188	10.5	10	29	529
B.S.	204–208	130	4.1	37	0.5	6	218	5.8	57	33	529
	259–263	115	4.9	33	.06	7	273	6.8	44	60	655
V.S.[2]	70–74	228	9.5	29	5.0	92	80	12.4	16	22	307

[1] 750 mg ascorbic acid per day.

[2] Owing to rapid failure of lactation the period of supplement for V.S. was only 5 days.

period with 1 exception. V.S. during the control period secreted milk with the highest concentration of the vitamin and excreted more than double the amount per day excreted by any other woman, yet after receiving 750 mg per day in addition to her diet for 5 days she excreted only 215 mg more in 24 hours than her average excretion per day during 5 days preceding supplement.

TABLE 2

Vitamin C in blood serum of mother and infant before and after supplement. (mg/100 ml.)

SUBJECT	CONTROL PERIOD			AFTER SUPPLEMENT		
	Interval postpartum	Mother [1]	Child	Interval postpartum	Mother	Child
	days			*days*		
V.G.	78	0.30	0.50	92	0.90	0.50
	161	0.21	1.72	175	1.04	1.40
	239	0.61 (0.96)	0.81	253	2.15	0.57
	302	0.16 (0.60)	1.07	316	1.31	1.19
V.K.	95	0.80	3.00	109	1.30	0.66
	144	0.73	0.92	158	1.34	0.73
V.L.	68	0.26	1.46	83	1.55	1.63
	152	0.80 (0.80)	1.43	170	1.39	1.59
J.M.	75	0.17	0.74	90	1.45	1.20
	172	0.20 (0.55)	0.64	188	1.19	0.84
B.S.	85	0.20	1.00	99	1.20	1.20
	204	0.16	0.79	218	0.94	0.90
	259	0.38	0.57	273	1.36	0.98
M.S.	58	0.04	0.63	72	1.12	1.21
V.S.	70	0.13 (0.36)	0.82	80	1.46	0.42

[1] Values in parentheses were obtained at the end of the 5-day control period. All other values represent the values on the first day of control period.

The estimations of vitamin C in the fasting samples of blood of the mothers and infants during the control period and following supplementation of the diet are given in table 2. Of the 15 values for blood obtained from the mothers on the first days of control periods, only 4 were above 0.6 mg per 100 ml, the amount regarded as comparab'e with health

(Smith, '38; Goldsmith and Ellinger, '39). The others were from 0.04 to 0.38 mg per 100 ml, an amount generally thought to indicate deficiency of vitamin C in the tissues. These results agree with those of Ingalls, Draper and Teel ('38) who found 0.3 mg per 100 ml on the fourteenth day postpartum. In 5 instances vitamin C was determined also in samples obtained after the mothers had received for 5 days the diet supplied during the control period. The blood level remained unchanged at 0.8 mg for 1 subject (V.L.) but the other 4 values showed increases of 0.35, 0.44, 0.35, and 0.23 mg.

After increasing the daily intake of vitamin C 4 to 8 times, the blood serum values of the women increased to within the range of saturation for non-pregnant non-lactating women (Storvick and Hauck, '42; Goldsmith and Ellinger, '39; Faulkner and Taylor, '38; Todhunter and Robbins, '40; Fincke and Landquist, '42), ranging from 0.9 to 2.2 mg per 100 ml of blood serum. The increases in concentration, with 1 exception, ranged from 1.6 to 11 times the values obtained the first day of the control period. The blood of M.S., was extremely low, 0.04 mg, at the start of the control period and increased to 1.12 mg following supplementation of her diet. Values after supplement were only 2 to 4 times greater than the determinations made following 5 days of eating the diet given during the control period.

DISCUSSION

Traversaro and Quesada ('38) have stated that the upper limit of vitamin C concentration in the blood and milk can be reached with food, or with crystalline vitamin C in the case of deficiency. Selleg and King ('36) observed that mothers with high initial vitamin C concentrations in milk showed relatively small increases when vitamin C supplements were given. It has been suggested (Traversaro and Quesada, '38; Baumann, '37) that there is an upper limit to the vitamin C secretion by the mammary gland, probably averaging between 7 and 8 mg per 100 ml. The data obtained in the present study support the belief in an upper limit of vitamin C concentra-

tion in human milk but emphasize the variability of that limit in different women.

Ingalls, Draper and Teel ('38) gave 300 and 600 mg vitamin C to nursing women during the first 2 weeks postpartum. After the first week the group receiving 600 mg of vitamin C per day had no greater concentration of vitamin C in their blood and milk than the group receiving 300 mg. They concluded that both groups had reached saturation at the end of the first week and that the excess vitamin was probably excreted in the urine. The ascorbic acid of the blood after 1 and 2 weeks rose to 1.38 and 1.44 mg per 100 ml in the supplemented groups. The milk values ranged from 5.0 to 9.5 and averaged 7.2 and 7.4 mg per 100 ml at the end of the first and second weeks, respectively. Selleg and King ('36) supplemented the diets of 2 groups of women with orange juice, equivalent to 210 and 430 mg of vitamin C per day during the first 10 days postpartum. The vitamin C concentration in the milk after supplementation rose to 7.3 and 8.1 mg per 100 ml on the tenth day. It would appear that the supplements given in these 2 studies served merely to bring the subjects' dietary intakes to an adequate level or to offset earlier poor nutritional state, for the values attained with the supplements compare with data presented from this laboratory (Munks, Robinson, Williams and Macy, '45) for the concentration of vitamin C in milk secreted during the first 10 days postpartum by healthy mothers receiving vitamin C only from a good diet of natural foods.

Widenbauer and Kühner ('37) determined for 6 mothers, 1 week to 16 months postpartum, the ascorbic acid in urine and milk per day during a preperiod and during a period of 20 to 27 days of oral administration of 400 to 500 mg of ascorbic acid per day. The subjects had very poor dietary histories and the study diets contained, besides potatoes and vegetables, small amounts of salad, but no fruit. When vitamin C excretion in urine rose a little, less vitamin C was given until excretion rose abruptly. The amount was then reduced until an approximate value for daily requirement

was reached. The ascorbic acid content of the breast milk was 0.5 to 2.2 mg per 100 ml before and 3.8 to 7.5 mg following supplementation. They comment that the values in the literature (4 to 7 mg per 100 ml) must have been obtained with milk from women who ate good diets with respect to vitamin C and conclude that their 6 women were vitamin C deficient to a greater or lesser extent as demonstrated by the saturation deficit. The daily ascorbic acid secretions in the milk before saturation were 5, 5, 4, 14, 13, and after supplementation were 10 mg and 60, 20, 18, 68, 78, 34 mg, respectively, increases to 3 to 11 times the earlier values, whereas the values reported in the present paper were only doubled. The daily excretions of the vitamin after supplement reported by Widenbauer and Kühner are within the range of the values in table 1 and indicate that the magnitude of increases in excretion of vitamin C supplements is dependent on the diets which the mothers have received for some time.

Widenbauer and Kühner concluded that the nursing mother requires 80 to 100 mg vitamin C per day in order to secrete 40 to 50 mg for the infant. From the data presented in this and the preceding paper it would seem necessary to supply the mother with considerably larger amounts, especially if based on values calculated from the literature, to assure the infant 40 to 50 mg per day. However, whether infants actually require that much vitamin C is not known. To eliminate the possibility of inadequate intakes of this important vitamin, common pediatric practice is the early addition of orange juice to even the breast-fed baby's diet.

It has been observed that there is a relatively slow and limited rise in the antiscorbutic value of breast milk when patients receive a special supplement of fruit juice equivalent to 210 or 430 mg of ascorbic acid per day. Sel'eg and King ('36) considered this noteworthy in that it provided an indication of a maximum and approximately optimum level of secretion, above which an excessive dietary intake results chiefly in a rapid urinary excretion without disturbing the lactation level. Other investigators (Chu and Sung, '36) found that a

sudden increase in the urinary excretion of vitamin did not occur until a large dose of vitamin had been given for 3 to 6 days, showing that the storage of vitamin C in the tissues of the mothers was very low at the beginning of the experiment. Once the peak was reached, the daily urinary output of vitamin C tended to be fairly constant, amounting to about 60% of the intake. They observed that excretion dropped abruptly with the discontinuance of the added vitamin.

Similar to the observation of Selleg and King, in a study of underfed mothers Chu and Sung ('36) observed that with an increase or decrease of vitamin C supply in the diet there was a corresponding fluctuation in the vitamin C content of milk, but the change was very slow and steady, being entirely different from the type of response in the urinary excretion. They suggest that "since milk is a product of secretion rather than excretion, it behaves like body tissue in this respect." After it had reached a saturation level, which was around 8 mg per 100 ml, the concentration of vitamin C in the milk remained high for about 10 days, even after the extra supply of the vitamin was discontinued.

The preceding paper emphasized the impossibility, at present, of distinguishing vitamin C storage from requirement for the substance. The same paper demonstrated that vitamin C in fasting blood samples could not be used as a criterion of the health of a nursing mother, the adequacy of her diet, or the vitamin C content of her milk and that there is a wide range of normal variation among healthy women. In the present study administration of 750 mg of ascorbic acid per day to healthy mothers eating good diets and successfully nursing their babies produced increases in the concentration of the vitamin in their milk and in fasting samples of their blood. However, these increases accounted for only a small fraction of the increases in intake, the greater portion of which was excreted in urine. During the control period the infants' blood levels (table 2) were much higher than their mothers', ranging from 0.50 to 3.00 mg per 100 ml. After the period of supplement the infants' blood contained 0.42

to 1.63 mg of vitamin C per 100 ml. In only 9 of 15 instances did the blood levels increase following ingestion of their mothers' milk with increased concentration. Without any evidence that the increased levels in the mothers' blood were beneficial to them, or that the increased concentrations in milk were helpful to their infants, the conclusion of the preceding paper seems justified: ". . . it seems that the Recommended Dietary Allowances ('45) of the Food and Nutrition Board of the National Research Council provide for adequate amounts of vitamin C to meet normal requirements during lactation and prevent depletion of body stores, provided during pregnancy the nutritional status of the mother has been adequate with respect to the vitamin."

SUMMARY

Values obtained by analysis for vitamin C in the milk, blood and urine of healthy mothers and in the blood of their breast-fed infants following administration of 750 mg of ascorbic acid per day for 6 to 14 days, have been compared with corresponding data for the same women and their infants during 5-day intervals immediately preceding the supplement period when analyses of the mothers' diets showed average intakes to be 102 to 228 mg of vitamin C per day.

Although the average levels of vitamin C in the breast milk and in fasting blood samples were higher following supplementation of the diet, the major portion of the ascorbic acid given was represented by increases in urinary excretion. The data suggest that the amount of vitamin C recommended by the Food and Nutrition Board for lactating women is sufficient if supplied by natural foods in a diet adequate with respect to other dietary essentials and if during pregnancy the nutritional status of the mother has been satisfactory.

LITERATURE CITED

BAUMANN, T. 1937 Untersuchungen über den C-Vitaminstoffwechsel bei laktierenden Frauen und über den Grad der physiologischen und pathologischen C-Vitamin sättigung des menschlichen Organismus. Jahrb. f. Kinderh., *150:* 193.

CHU, F. T., AND C. SUNG 1936 Effect of vitamin C administration on vitamin C of milk and urine of lactating mothers. Proc. Soc. Exp. Biol. Med., *35:* 171.

DAVIES, V. 1945 Human milk studies XXI. A simple technic for the manual expression of mothers' milk. Am. J. Dis. Child., *70:* 148.

FARMER, C. J., AND A. F. ABT 1936 Determination of reduced ascorbic acid in small amounts of blood. Proc. Soc. Exp. Biol. Med., *34:* 146.

FAULKNER, J. M., AND F. H. L. TAYLOR 1938 Observations on the renal threshold for ascorbic acid in man. J. Clin. Invest., *17:* 69.

FINCKE, M. L., AND V. L. LANDQUIST 1942 The daily intake of ascorbic acid required to maintain adequate and optimal levels of this vitamin in blood plasma. J. Nutrition, *23:* 483.

FOOD AND NUTRITION BOARD 1945 Recommended Dietary Allowances. National Research Council Reprint and Circular Series No. 122.

GOLDSMITH, G. A., AND G. F. ELLINGER 1939 Ascorbic acid in blood and urine after oral administration of a test dose of vitamin C. A saturation test. Arch. Int. Med., *63:* 531.

INGALLS, T. H., R. DRAPER AND H. M. TEEL 1938 Vitamin C in human pregnancy and lactation. II. Studies during lactation. Am. J. Dis. Child., *56:* 1011.

KAUCHER, M., E. Z. MOYER, A. J. RICHARDS, H. H. WILLIAMS, A. L. WERTZ AND I. G. MACY 1945 Human milk studies. XX. The diet of lactating women and the collection and preparation of food and human milk for analysis. Am. J. Dis. Child., *70:* 142.

KAUCHER, M., E. Z. MOYER, H. H. WILLIAMS AND I. G. MACY 1946 Adequacy of the diet during lactation. J. Am. Diet. Assoc., *22:* 594.

MACY, I. G., H. H. WILLIAMS, J. P. PRATT AND B. M. HAMIL 1945 Human milk studies. XIX. Implications of breast feeding and their investigation. Am. J. Dis. Child., *70:* 135.

MUNKS, B., M. KAUCHER, E. Z. MOYER, M. E. HARRIS AND I. G. MACY 1947 Metabolism of women during the reproductive cycle. XI. Vitamin C in diets, breast milk, blood and urine of nursing mothers. J. Nutrition, *33:* 601.

MUNKS, B., A. ROBINSON, H. H. WILLIAMS AND I. G. MACY 1945 Human milk studies. XXV. Ascorbic acid and dehydroascorbic acid in colostrum and mature human milk. Am. J. Dis. Child., *70:* 176.

RALLI, E. P., G. J. FRIEDMAN AND S. SHERRY 1939 The vitamin C requirement of man, estimated after prolonged studies of the plasma concentration and daily excretion of vitamin C in 3 adults on controlled diets. J. Clin. Invest., *18:* 705.

ROE, J. H., AND C. A. KUETHER 1943 The determination of ascorbic acid in whole blood and urine through the 2,4 dinitrophenyl-hydrazine derivative of dehydroascorbic acid. J. Biol. Chem., *147:* 399.

SELLEG, I., AND C. G. KING 1936 The vitamin C content of human milk and its variation with diet. J. Nutrition, *11:* 599.

SMITH, S. L. 1938 Human requirements of vitamin C. J. Am. Med. Assoc., *111:* 1753.

STORVICK, C. A., AND H. M. HAUCK 1942 Effect of controlled ascorbic acid ingestion upon urinary excretion and plasma concentration of ascorbic acid in normal adults. J. Nutrition, *23:* 111.

TODHUNTER, E. N., AND R. C. ROBBINS 1940 Observations on the amount of ascorbic acid required to maintain tissue saturation in normal adults. J. Nutrition, *19:* 263.

TRAVERSARO, J. C., AND R. QUESADA 1938 Vitamina C en la leche de mujer. Rev. Assoc. Bioquim. Argentina, *3:* 7.

WIDENBAUER, F., AND A. KÜHNER 1937 Ascorbinsäurestudien an stillenden Frauen. Zeitschr. f. Vitaminfors., *6:* 50.

BLOOD LEVELS AND ABSORPTION OF VITAMIN A IN CHILDREN WITH KERATOSIS FOLLICULARIS [1]

R. F. KRAUSE AND HAROLD B. PIERCE

WITH THE TECHNICAL ASSISTANCE OF

RUTH LAW AND HATTIE KAPLAN

Department of Biochemistry, College of Medicine, University of Vermont, Burlington

TWO FIGURES

(Received for publication February 17, 1947)

It has been postulated by Peck et al. ('43) that keratosis follicularis is a deficiency syndrome which may be caused by either a hereditary or an acquired weakness in absorption of vitamin A or in conversion of provitamin A to vitamin A. In 10 cases of this disease he reports 8 with the vitamin A content of the blood below "normal limits," and 2 at the lower limits of "normal." Carelton and Steven ('43) and Cornbleet et al. ('44) report a total of 5 cases of this disease with no evidence of vitamin A deficiency in the blood.

In view of these different findings it was believed worthwhile to investigate, first, if individuals with folliculosis do have abnormally low vitamin A and carotene serum levels and secondly, if these individuals exhibit a weakness in the absorption of vitamin A.

METHODS AND PROCEDURES

Children were selected for study since Pierce et al. ('45) found a high incidence of folliculosis among Burlington chil-

[1] The work was supported in part by grants from Merck and Company, Inc., Milbank Memorial Fund and National Vitamin Foundation, Inc.

dren. Children from Burlington Public and Parochial schools and from St. Joseph's Orphanage were examined for the presence of folliculosis.[2] Two hundred and fifteen were chosen for the folliculosis group and 179 for the non-folliculosis group. Finger-tip blood was taken from each child at the time of the physical examination for the determination of vitamin A and carotene by the methods of Bessey et al. ('46b).

Vitamin A absorption studies were made on 36 Orphanage children between the ages of 8 and 15 years. Nineteen of these children had folliculosis and were used as a "test group" and 17 without folliculosis were used as a "control group." Vitamin A capsules[3] containing 50,000 I.U. were given to 12 "test" and 12 "control" children and 25,000 I.U. to 7 "test" and 5 "control" children. Finger-tip blood was taken prior to the vitamin administration and 4, 5, 6, 8 and 24 hours thereafter, for vitamin A and carotene determinations.

No effort was made to control the diet of these children since it has been shown that ordinary mixed meals have little or no effect upon the concentration of either vitamin A or carotene in serum (Kimble, '39). Then too, if any change did occur in serum levels due to food ingested it would be relatively constant for all children since they were institutionalized and eating the same food.

RESULTS

Table 1 summarizes the data obtained from the determination of serum vitamin A and carotene values of children with and without folliculosis. The average vitamin A concentrations for both groups of children were the same, namely 38 µg %, whereas the average carotene values were significantly lower in the group with folliculosis.

[2] The extremities were examined for the presence of keratosis follicularis by Drs. John H. Browe, Research Associate in Medicine; C. A. Newhall, Assoc. Prof. Anatomy; T. H. Harwood, Asst. Prof. Medicine and P. D. Clark, Asst. Prof. Pediatrics, Medical College, University of Vermont.

[3] The vitamin A used in this study was supplied by Eli Lilly and Company, Indianapolis, Ind.

TABLE 1

Serum concentration of vitamin A and carotene of children with and without folliculosis.

	CHILDREN WITH FOLLICULOSIS		CHILDREN WITHOUT FOLLICULOSIS	
	Vitamin A	Carotene	Vitamin A	Carotene
Number of children	215	215	179	179
Micrograms per cent	38	101	38	116
Standard deviations	± 9.8	± 16.5	± 10.3	± 45.7

TABLE 2

Vitamin A serum levels of test and control children after oral administration of 50,000 I.U. of vitamin A.

SUBJECT	BASAL	HOUR AFTER VITAMIN A ADMINISTRATION				
		4	5	6	8	24
			µg % vitamin A in serum			
				Control Group		
1	35	141	187	88	58	42
2	32	51	78	81	44	47
3	27	98	128	69	75	41
4	27	61	85	53	47	36
5	26	62	129	138	..	40
6	28	33	40	51	54	39
7	30	43	194	88	74	38
8	26	90	196	111	46	28
9	19	37	159	120	41	34
10	19	29	84	100	47	31
11	36	81	114	95	58	29
12	27	81	91	50	39	21
Average	27.6	67.2	123.8	87.0	52.9	35.5
St. dev.	± 5.4	± 31.2	± 49.0	± 26.9	± 11.6	± 6.9
				Test Group		
13	29	84	183	122	67	36
14	30	54	80	56	86	48
15	34	67	212	122	74	66
16	27	21	48	30	28	37
17	46	91	118	75	60	71
18	31	26	88	86	80	40
19	26	81	171	126	86	26
20	18	46	126	107	98	25
21	26	43	171	156	75	38
22	23	12	39	45	47	32
23	27	37	152	97	40	35
24	19	48	100	121	67	34
Average	28.0	50.8	123.9	95.5	67.3	40.6
St. dev.	± 6.9	± 10.9	± 20.8	± 13.1	± 6.7	± 4.4

TABLE 3

Vitamin A serum levels of test and control children after oral administration of 25,000 I.U. of vitamin A.

SUBJECT	BASAL	HOUR AFTER VITAMIN A ADMINISTRATION				
		4	5	6	8	24
				µg % vitamin A in serum		
				Control Group		
25	20	103	41	49	22	18
26	28	77	83	78	71	33
27	15	86	105	118	51	26
28	28	37	64	55	..	24
29	37	39	48	41	39	38
Average	26	68	68	68	45	28
St. dev.	± 7.6	± 26.2	± 23.4	± 27.8	± 17.9	± 7.0
				Test Group		
30	33	50	58	..	46	32
31	24	13	22	34	38	30
32	15	23	66	44	34	15
33	29	59	57	50	35	31
34	22	88	87	65	32	27
35	23	31	90	46	26	21
36	21	26	97	150	58	22
Average	24	41	68	65	38	25
St. dev.	± 5.4	± 24.0	± 20.8	± 39.2	± 9.8	± 5.8

In the absorption study, the basal vitamin A levels and those for 4, 5, 6, 8 and 24 hours after the administration of vitamin A are presented in tables 2 and 3. The average rise in serum vitamin A and the ensuing average drop for each interval of time after the ingestion are noted, for both the "test" and "control" groups, in figures 1 and 2, and the resulting curves are designated as average "tolerance" curves.

In all but 2 cases the maximum rise in serum concentration was reached on either the fifth or sixth hour after ingestion.

Fig. 1 The average "tolerance" curves for 12 children with folliculosis (Test group) and 12 children without folliculosis (Control group) after the ingestion of 50,000 I.U. of vitamin A.

Fig. 2 The average "tolerance" curves for 5 children without folliculosis (Control group) and 7 children with folliculosis (Test group) after the ingestion of 25,000 I.U. of vitamin A.

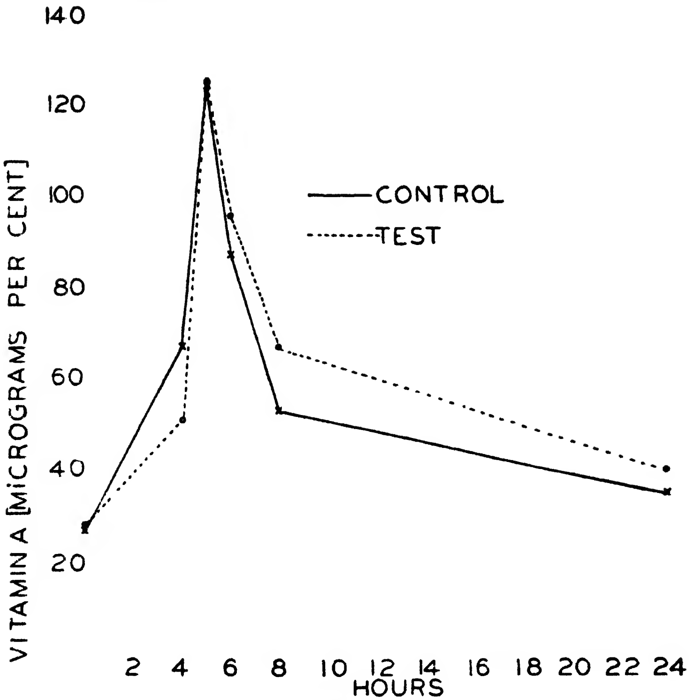


Figure 1

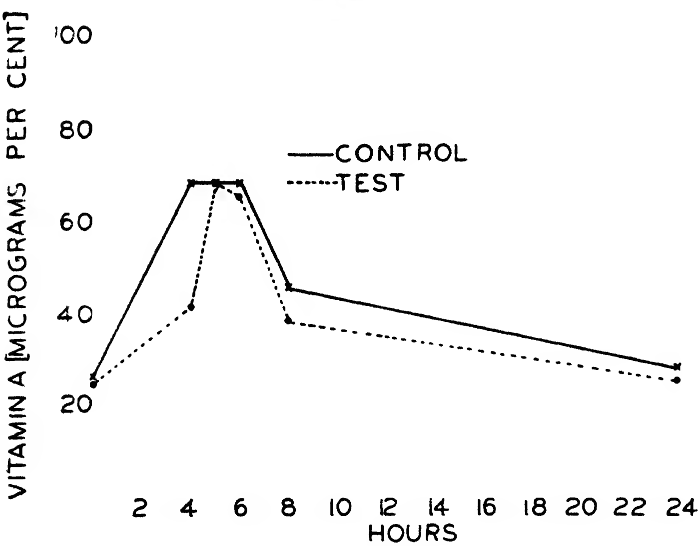


Figure 2

TABLE 4

Serum carotene levels of test and control children after oral administration of 50,000 and 25,000 I.U. of vitamin A.

SUBJECT	BASAL	HOUR AFTER VITAMIN A ADMINISTRATION				
		4	5	6	8	24
		µg % vitamin A in serum				
		Control Group				
		After 50,000 I.U. of vitamin A				
1	74	69	78	67	68	72
2	79	79	75	76	76	76
3	91	89	96	92	95	89
4	61	57	63	61	61	64
5	64	61	61	65	..	68
6	67	65	66	62	67	62
7	90	86	85	84	89	86
8	82	78	75	75	74	79
9	109	106	95	100	104	107
10	84	88	79	80	84	84
11	120	124	112	110	113	110
12	88	88	83	80	85	84
		After 25,000 I.U. of vitamin A				
25	75	75	70	73	69	70
26	85	87	84	85	83	80
27	50	55	51	55	53	51
28	54	54	51	50	53	50
29	32	32	30	29	30	33
		Test Group				
		After 50,000 I.U. of vitamin A				
13	62	64	59	58	62	68
14	77	75	74	74	80	74
15	80	79	85	83	79	83
16	87	86	86	86	90	86
17	80	78	78	80	78	82
18	55	56	60	55	52	54
19	56	54	54	55	54	56
20	94	100	94	92	94	94
21	60	62	62	61	58	59
22	62	59	54	58	60	59
23	75	71	71	70	70	73
24	77	76	73	73	75	77
		After 25,000 I.U. of vitamin A				
30	31	32	28	..	31	29
31	54	60	54	57	54	58
32	28	29	27	27	28	31
33	57	56	54	54	60	55
34	66	59	58	57	57	55
35	79	85	81	77	80	81
36	43	47	44	45	46	44

This maximum rise was, on the average, about 4½ and 2½ times higher than the average basal values for the 50,000 and 25,000 I.U. dosages, respectively. At the end of 8 hours the concentration was reduced to one-half its peak value and by 24 hours was back to normal for the groups receiving 25,000 I.U. but was still slightly elevated for the group receiving the higher dosage.

The basal carotene levels and those for 4, 5, 6, 8 and 24 hours after administration of vitamin A are shown in table 4.

DISCUSSION

It is evident from the data presented in table 1 that the children in this study who have folliculosis do not have lower vitamin A serum levels than "normal" children. It is interesting to note, however, that the serum carotene concentration was lower in children with folliculosis. No information is available for an explanation of this finding.

Bessey ('46a) has stated that, from his experiences, the normal serum vitamin A and carotene levels range between 30–70 μg % and 100–300 μg %, respectively. In this study the average serum vitamin A and carotene values for both groups of children lie within the lower limits of these ranges.

It may be noted in tables 2 and 3 that the concentration of vitamin A in the serum at any one time after the ingestion of this vitamin is variable from child to child and that the rate and magnitude of these changes are not necessarily related to the basal vitamin A levels[4] or to the presence or absence of folliculosis. The average "tolerance" curves for children with and without folliculosis are not significantly different. In view of these findings it is concluded that the children in this study who had folliculosis did not exhibit a weakness in absorption of vitamin A.

The serum carotene levels were not affected by the dosages of vitamin A ingested and remained essentially constant throughout the 24-hour absorption study period (see table 4).

[4] Steigmann and Popper ('44) have reported similar findings in their study entitled "The influence of large doses of vitamin A upon the plasma vitamin A level."

These data present additional confirmatory evidence for the fact that large doses of vitamin A do not alter the concentration of carotene in serum; and that the reaction, carotene → vitamin A appears to be an irreversible one (Peters and Van Slyke, '46).

SUMMARY

1. In a survey of 394 children no significant difference was found between the serum vitamin A levels of children with and without folliculosis.

2. The serum carotene values were lower in children with folliculosis.

3. The children of both groups are equally capable of absorbing vitamin A as indicated by their serum levels after the administration of 50,000 and 25,000 I.U. of vitamin A.

ACKNOWLEDGMENT

The authors wish to express their thanks for the assistance and cooperation given this study by the personnel of St. Joseph's Orphanage and Burlington's Public and Parochial Schools.

LITERATURE CITED

BESSEY, O. A. 1946 Personal communication.

BESSEY, O. A., O. H. LOWRY, M. J. BROCK AND J. A. LOPEZ 1946b The determination of vitamin A and carotene in small quantities of blood serum. J. Biol. Chem., *166:* 177.

CARLETON, A., AND D. STEVEN 1943 Keratosis Follicularis. Arch. Derm. and Syph., *48:* 143.

CORNBLEET, T., H. POPPER AND F. STEIGMANN 1944 Blood vitamin A and cutaneous diseases. Arch. Derm. and Syph., *49:* 103.

KIMBLE, M. S. 1939 The photocolorimetric determination of vitamin A and carotene in human plasma. J. Lab. Clin. Med., *24:* 1055.

PECK, S. M., A. W. GLICK, H. H. SOBOTKA AND L. CHARGIN 1943 Vitamin A studies in cases of Keratosis Follicularis (Darier's Disease). Arch. Derm. and Syph., *48:* 17.

PETERS, J. P., AND D. D. VAN SLYKE 1946 Quantitative Clinical Chemistry, *1:* 611.

PIERCE, H. B., P. F. FENTON, W. WILKINS, M. E. NEWLAND AND P. S. BUDGE 1945 Nutritional defects among children in Vermont. New Eng. J. Med., *233:* 612.

STEIGMANN, F., AND H. POPPER 1944 The influence of large doses of vitamin A upon the plasma vitamin A level. Am. J. Med. Sci., *207:* 468.

THE EFFECT OF FAT LEVEL OF THE DIET ON GENERAL NUTRITION

III. WEIGHT LOSS, MORTALITY AND RECOVERY IN YOUNG ADULT RATS MAINTAINED ON RESTRICTED CALORIES [1]

BRADLEY T. SCHEER, JOHN F. CODIE AND H. J. DEUEL, JR.
Department of Biochemistry and Nutrition, University of Southern California, School of Medicine, Los Angeles

(Received for publication January 2, 1947)

Previous papers have described the effects of feeding diets varying in fat content ad libitum, and in restricted amounts, to weanling rats (Deuel et al., '47; Scheer et al., '47a). In the experiments to be reported here, rats were maintained on a stock diet for 19 weeks after weaning, and then transferred to the experimental diets, fed in restricted amounts. Measurements of body weight and physical capacity were made during the period of caloric restriction and a subsequent recovery period of ad libitum feeding; reproductive performance was also tested in the recovery period. The methods used have been described earlier (Deuel et al., '47; Scheer et al., '47b). During the present work the restricted food portions were fed daily, instead of every second day, as in the earlier experiments. Three hundred rats were divided into groups at weaning and kept in large cages in groups of 4 for 19 weeks. At the end of

[1] The subject matter of this paper has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

We are indebted to Merck and Co., Rahway, N. J., for the synthetic B vitamins; to the Winthrop Chemical Co., Albany, N. Y., for the crystalline vitamin D_2 and to Lederle Laboratories, Inc., Pearl River, N. Y., for the folic acid.

this time, they were transferred to smaller individual cages and fed the experimental diets in restricted amounts.

RESULTS

Weight loss

On the basis of a preliminary experiment, in which young adult rats were maintained on the stock diet at various levels of caloric intake for 6 weeks, it was calculated that an intake of 24 cal. per day would result in a weight loss of 50% in 12 weeks. After 8 weeks on the experimental regime, the weights of the animals receiving the diets with various amounts of fat at a level of 24 cal. per day were greater than had been predicted (table 1); we accordingly reduced the caloric intake to 12 cal. per day. After a total experimental period of 12 weeks, the males had lost 40 to 50% of their initial weight, while the females had lost 30 to 40%.

Table 1 presents the mean weights with their standard errors at intervals of 4 weeks during the restricted period. It is apparent that the greatest weight loss was observed on diet 60b, containing no fat but with 1% methyl linolate, while the smallest losses were observed on diet 63, containing 20% fat, and the stock diet, containing 14% fat. Those differences which might have occurred by chance less than once in 100 samples (difference/standard error > 2.5) are also tabulated in table 1. The results presented appear to justify the conclusion that loss of weight under conditions of severe caloric restriction is greater when the diet contains 10% of fat or less than when it provides 15 to 40% of fat. However, there is no evident advantage of the 40% level over 20%. The apparent superiority of the stock diet during the last weeks of the experiment resulted from a failure of appetite in the other groups, as will be noted later.

Mortality

In earlier work, we noted that mortality among weanling rats fed restricted calories was highest on diet 61 (5% fat) and least on the stock diet (14%) and diet 64 (40%). A very

TABLE 1

Weights of young adult rats fed experimental diets in amounts to provide 24 cal. per day for 8 weeks, followed by 12 cal. per day for 4 weeks.

DIET NO.	FAT CONTENT IN %	BODY WEIGHT IN GM[1]				M.D. : S.E.M.D.[2]	
		Start	4 wks	8 wks.	12 wks	8-wk. period	12-wk. period
				Male rats			
60B	0[3]	269.5 ± 5.4	220.7 ± 1.5	200.1 ± 3.8	126.5 ± 4.5	62, 3.0; 63, 3.9; S., 3.1.	63, 3.4; 64, 3.2.
61	5	264.9 ± 2.0	223.2 ± 3.7	211.8 ± 3.9	128.0 ± 4.6		63, 3.1; 64, 2.9.
62	10	274.9 ± 5.7	235.2 ± 3.6	218.4 ± 4.7	134.6 ± 3.9		63, 2.5.
63	20	267.4 ± 7.1	225.7 ± 4.8	216.7 ± 2.0	147.9 ± 4.4		
64	40	263.1 ± 6.2	215.2 ± 5.2	201.3 ± 4.6	142.6 ± 12.1	62, 2.6; 63, 3.1.	
S[4]	14	268.4 ± 4.5	224.6 ± 2.9	213.7 ± 2.3	145.6 ± 6.5		
				Female rats			
60B	0[3]	183.4 ± 3.2	163.3 ± 2.6[5]	164.1 ± 1.4	116.2 ± 5.5	61, 3.5; 62, 4.0; 63, 5.2; S., 3.8.	S., 3.1.
61	5	189.2 ± 5.7	168.9 ± 1.2	171.6 ± 1.9	105.0 ± 2.5		63, 5.0; S., 8.5.
62	10	188.7 ± 3.1	176.0 ± 2.0	180.3 ± 2.0	113.8 ± 3.5	60B, 4.0.[5]	S., 4.9.
63	20	182.9 ± 3.6	169.3 ± 2.4	177.7 ± 2.2	124.7 ± 3.1		S., 2.5
64	40	188.6 ± 3.4	167.3 ± 2.6	169.7 ± 2.3	121.5 ± 7.6		
S[4]	14	189.8 ± 8.9	171.6 ± 3.1	174.1 ± 2.2	134.6 ± 2.4		

[1] Including the standard error of the mean calculated as follows $= \sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

[2] Ratio of mean difference to standard error of mean difference. When this is 2.5, it indicates that the chances that such a difference might arise by chance are no more than 1 to 100.

[3] Containing 1.0% methyl linolate.

[4] Stock diet.

[5] Ratio M.D. : S.E.M.D. for 4-week period.

similar distribution of mortality among the groups was noted in this study (table 2). In the present experiment, death was often preceded by appearance of bloody incrustations on the coat, and lack of appetite. The bloody coats were especially evident on the abdomen and forelimbs in females; the source of the discoloration was not certain although nasal bleeding was observed in a few instances. A short time after this phenomenon was noted, some of the animals failed to consume their daily food portion completely. This lack of appetite appeared first on diet 61 (5%), then on 62 (10%) and 60 (0%), and finally on 63 (20%) and 64 (40%). The animals fed the stock diet retained a vigorous appetite throughout the experiment. Finally during the last weeks of the experiments, animals began to die. This was not always simply the result of failure to eat, since a number of animals continued to eat regularly until they died. In other cases, however, animals failed to eat even after transfer to ad libitum feeding in large cages. These animals died during the thirteenth week (table 2).

There seems to be no obvious explanation for these observations. The lack of appetite suggests a deficiency of thiamine, but the diets used have supported excellent growth, reproduction and lactation when fed ad libitum. The superiority of the stock diet suggests the possibility that vitamins were present in the natural foods (wheat, oats, yeast, alfalfa) comprising this diet, or were synthesized by intestinal flora, which were not available to animals on the synthetic diets. It is possible that, under the special conditions of this experiment, significant alterations in intestinal synthesis might appear. We have often noted transient loss of hair on the head and shoulders, suggesting biotin deficiency, after transfer from restricted to ad libitum feeding. Experiments are now in progress to test these points further.

The results of the tests of physical capacity have been presented elsewhere (Scheer et al., '47a). They add no further illuminating details to the general picture; a marked decrease in endurance appeared in all groups but was not related to

fat content in the diet. Recovery was rapid and complete in all groups.

TABLE 2

The total mortality, bleeding and lack of appetite in young adult rats fed experimental diets at levels of 24 cal. per day for 8 weeks and 12 cal. per day for following 4 weeks.

WEEKS ON RESTRICTED DIET	RESULTS AFTER FOLLOWING DIETS									
	60B		61		62		63		64	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
				Total mortality						
10				4						
11	4		8	4	4	4			4	
12	35	21	67	32	56	29	24	16	4	4
13	65	58	75	44	70	50	52	36	12	20
				Bleeding						
7						4				
8				12		13				
9				16		22				
10				12	4	22		4		
11			9	12	13	18	13	8		
				Loss of appetite						
8			25	52						
9			29	52						
10	9	37	92	96	50	69	4			8
11	77	84	100	100	96	100	87	100	13	28
12	94	100	100	100	100	100	100	100	35	68

Recovery of body weight

At the end of 12 weeks, most of the surviving animals were returned to large cages and fed the same diets ad libitum. Several from each group, however, were sacrificed for determinations of body composition. The mean weights of the survivors with their standard errors, are presented in table 3. The high mortality, especially among the males, reduced the number of animals involved; consequently the differences are not always statistically significant; those for which the probability of a similar result by chance is less than 1 in 100 are tabulated at the right of the table. The superiority of

TABLE 3

Changes in weight of young adult rats surviving a 12-week period of severe caloric restriction during a subsequent period of ad libitum feeding.

DIET NO.	NO. OF RATS	BODY WEIGHT IN GM [1]					M.D. : S E.M.D.[2]	
		Start	After 12 wks. restricted	Recovery period			3-wk. recovery	6-wk. recovery
				3 wks.	6 wks	9 wks.		
Male rats								
60B [3]	4	273.0 ± 15.9	140.2 ± 8.5	173.2 ± 53.2	242.9 ± 12.9			63, 3.7; S., 2.5.
61	1	296	128	172	247	277		
62	1	250	130	248	283			
63	6	274.0 ± 12.2	159.7 ± 7.3	259.5 ± 8.3	304.7 ± 10.5	303.7 ± 12.9		
64	12	260.9 ± 10.2	140.4 ± 5.0	237.4 ± 8.0	291.6 ± 5.3			
S [4]	15	267.5 ± 5.6	147.7 ± 8.7	244.1 ± 6.0	286.9 ± 11.9			
Female rats								
60B [3]	7	187.9 ± 7.2	121.0 ± 4.0	171.4 ± 7.1			63, 4.8; 64, 3.3; S., 4.0.	
61	6	192.8 ± 7.5	112.8 ± 5.5	184.8 ± 4.4	212.7 ± 5.3	213.5 ± 7.9	63, 4.4; S., 3.1.	63, 2.7.
62	6	187.0 ± 8.2	122.2 ± 13.8	177.2 ± 10.0	203.2 ± 13.3	213.0 ± 16.6	63, 3.2; S., 2.5.	
63	10	191.1 ± 4.9	133.6 ± 4.9	211.5 ± 4.2	235.0 ± 6.2		61, 2.8.[5]	64, 2.6; S., 3.6.
64	13	192.1 ± 3.7	123.1 ± 3.7	198.5 ± 4.1	212.6 ± 5.8	213.6 ± 5.9		
S [4]	20	190.2 ± 3.5	134.3 ± 2.2	205.0 ± 4.7	205.4 ± 5.3	217.5 ± 6.2	60B, 4.5; 61, 3.6; 64, 2.6.[5]	

[1] Including the standard error of the mean calculated as follows $= \sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

[2] Ratio of mean difference to standard error of mean difference. When this is 2.5, it indicates that the chances that such a difference might arise by chance are no more than 1 to 100.

[3] Containing 1.0% methyl linolate.

[4] Stock diet.

[5] Ratio M.D. : S.E.M.D. for 12-week restricted period.

diet 63 (20% fat) in supporting recovery of body weight is evident in the female series. It is of interest to note here that the stock diet, which supported rapid gain in weight to a value slightly above the weight at the beginning of the experiment, did not permit as great eventual gains as did diet 63.

Reproductive performance

Immediately after transfer to ad libitum feeding, 5 females from each dietary group were placed in separate large cages with males from the same group if these were available. Only 1 litter was born to animals subsisting on diets 60b and 62 and none on diet 61 while 5 litters were produced each by the females on diets 63 and 64 and 4 on the stock diet. None of the litters was successfully raised to weaning in spite of the fact that the number of young per litter was small. The results are in sharp contrast with those presented earlier (Scheer, '47b) in which most of the litters born to rats which had been subjected to caloric restriction immediately after weaning were reared successfully. Evidently recovery of reproductive capacity after such severe restriction is much less rapid than recovery of body weight or physical capacity.

DISCUSSION

The experiments presented in this series of papers (Deuel et al., '47; Scheer et al., '47a, '47b) have invariably resulted in the same conclusions; in every case, animals performed more satisfactorily when fed diets containing liberal amounts of fat than when fed diets containing little or no fat. It is possible to explain the growth effects with ad libitum feeding on the basis of increased caloric intake with higher fat content of the diet. The results with restricted isocaloric feeding cannot be so explained. The valuable observation of Forbes et al. ('46a, '46b) that the metabolic efficiency of food utilization is greater when fat is present suggests one important possible explanation. It does not seem however, that all of the effects which we have observed, i.e., on physical capacity, resistance to undernutrition, reproduction and lactation, can be the result

simply of increased efficiency of food utilization. Rather, it appears that fat must take an active part in a variety of fundamental vital processes underlying the phenomena which we have observed. Increased fat in the diet, then, would be expected to increase the effectiveness of all of these processes by providing a readily available stock of fat, thus obviating the relatively expensive necessity of fat formation from carbohydrate. In this view, fat is no longer the inert storage material of the older biochemistry, but an active component of the metabolic machinery.

SUMMARY

When young adult rats are subjected to severe caloric restriction on diets varying in fat content, weight loss is less rapid, and mortality is less, on diets containing liberal amounts of fat than on a fat-free diet. During recovery from the period of caloric restriction, weight loss is more rapidly regained, and reproductive capacity is superior on diets containing fat. Reproductive capacity is still subnormal 4 to 6 weeks after transfer to ad libitum feeding, a time when body weight and physical capacity have returned to normal. The significance of the results of the present series of experiments is briefly discussed in relation to the role of fat in metabolism.

LITERATURE CITED

DEUEL, H. J., JR., E. R. MESERVE, E. STRAUB, C. HENDRICK AND B. T. SCHEER 1947 The effect of fat level of the diet on general nutrition. I. Growth, reproduction and physical capacity of rats receiving diets containing various levels of cottonseed oil or margarine fat ad libitum. J. Nutrition, *33:* 569.

FORBES, E. B., R. W. SWIFT, R. F. ELLIOTT AND W. H. JAMES 1946a Relation of fat to economy of food utilization. I. By the growing albino rat. J. Nutrition, *31:* 203.

———— 1946b Relation of fat to economy of food utilization. II. By the mature albino rat. J. Nutrition, *31:* 213.

SCHEER, B. T., S. DORST, J. F. CODIE AND D. F. SOULE 1947a Physical capacity of rats in relation to the energy and fat content of the diet. Am. J. Physiol. (in press).

SCHEER, B. T., D. F. SOULE, M. FIELDS AND H. J. DEUEL, JR. 1947b The effect of fat level of the diet on general nutrition. II. Growth, mortality and recovery in weanling rats maintained on restricted calories. J. Nutrition, *33:* 583.

NUTRITIONAL SURVEYS IN WESTERN HOLLAND: ROTTERDAM, 1945 [1]

CYRUS E. FRENCH [2] AND FREDRICK J. STARE [3]

Institute of Animal Nutrition, The Pennsylvania State College, State College, Pa.; and Department of Nutrition, Schools of Public Health and Medicine, Harvard University, Boston, Massachusetts

ONE FIGURE

(Received for publication February 8, 1947)

INTRODUCTION

During the course of the war the Nutrition Division, Office of the Surgeon General, U.S. Army, under the direction of Col. John B. Youmans, formulated detailed plans for nutritional surveys of civilian populations in liberated and occupied countries. When the defeat of the German armies in the Netherlands appeared imminent early in March, 1945, a survey team was requested by the Public Health Branch of Supreme Headquarters Allied Expeditionary Forces and was immediately dispatched to that area. This team joined a similar Canadian group headed by Wing Commander J. M. McCreary in performing a series of 1-day surveys of newly liberated cities and towns during the advance into southern Holland with the First Canadian Army. These rapid surveys were designed to furnish Civil Affairs detachments with immediate information about local food supplies and nutritional conditions.

[1] Presented at the meetings of the Agricultural and Food Division, American Chemical Society at Chicago, September 9–12, 1946.

[2] On leave; Captain, Sn.C., Army of the United States.

[3] Consultant to the Surgeon General, United States Army.

Each survey team consisted essentially of the following: a medical officer with special training in deficiency diseases; a nutrition officer with special training in obtaining dietary histories, and in conducting biochemical studies; a senior Dutch medical student who acted as interpreter and assistant; and clerical personnel. The American team was equipped with a portable laboratory designed and constructed by the senior author with the cooperation of Drs. R. E. Johnson ('45), O. A. Bessey and O. H. Lowry ('45), for making certain laboratory studies in the field such as hemoglobin, serum protein, plasma ascorbic acid, and urinary excretion tests for thiamine, riboflavin, nicotinic acid, chlorides, albumin and ketone bodies. It proved impractical however to attempt laboratory investigations in connection with the rapid surveys because of the emphasis on speed and movement. When detailed surveys were carried out in the cities of western Holland much biochemical work was done.

The First Canadian Army was moving northward into central Netherlands against very determined resistance during April and early May, 1945. The British Second Army meanwhile had swung to the east and moved rapidly northward along the Dutch German border, thus effectively cutting off a large number of German forces from their bases of supply. As the German forces and large numbers of inhabitants were steadily forced back into the smaller, more densely populated, and less agriculturally productive area of western Holland reports of hunger and starvation in the civilian population were received in ever increasing numbers. Although unconfirmed, such reports seemed logical in view of the general overcrowded condition and the customary practice of German troops to live off the land. Accordingly, detailed plans and procedures of operation for the assessment of nutritional conditions and possible relief measures were formulated with Dutch, British and American authorities by the nutrition branch of SHAEF Mission Netherlands under the direction of Lt. Col. H. R. Sandstead, U.S. Public Health Service.

Following the capitulation of the German military forces in western Holland on May 7, 1945, similar rapid surveys were made in that area to determine the immediate needs of the population for emergency food supplies, medical care and for aid from Netherlands Red Cross feeding teams. In general only poor sections of the cities were sampled in these preliminary studies and nutritional conditions were found to be much more severe than in southern and eastern Holland. By the time these studies were completed on May 18, 1945, conditions had improved considerably. Emergency food supplies were being distributed and Netherlands Red Cross feeding teams were setting up and operating a number of emergency hunger wards in many of the hospitals.

The Canadian and American survey teams were then joined by a British unit and mobile laboratory headed by Dr. H. M. Sinclair and each of the teams was augmented with Dutch physicians, biochemists, dietists, secretarial and other personnel. Detailed surveys were then undertaken in Leiden and The Hague by the British team, in Utrecht and Amsterdam by the Canadian team and in Delft and Rotterdam by the American team. Nearly 14,000 persons of all ages and economic groups were surveyed in these comprehensive studies which were completed by the end of June, and definite evidence of widespread starvation was recorded (Burger et al., '45). Results were used to advise allied authorities regarding the kinds and quantities of food needed during the period of recuperation; to advise and support Dutch food and health authorities; and to determine the effects of extended underfeeding on the entire population. With reference to the latter purpose it should be mentioned that a monograph is being prepared by Dutch, British, Canadian and American investigators which will constitute a comprehensive scientific report of the causes, the course of events, and the results of mass starvation in Holland.

The data to be presented and discussed in this report will be limited to those obtained in surveys of Rotterdam on May 9 and again during the period May 29 to June 8, 1945. They may

be considered to be representative generally of conditions that existed at this time in the other great cities of western Holland as judged by the findings of the other teams. Rotterdam is the principal seaport and third largest city of the Netherlands. The normal population of 600,000 remained unchanged despite the severe German bombings in 1940 that destroyed large areas in the heart of the city and that resulted in considerable overcrowding and unemployment. It was selected for a detailed investigation following the rapid survey of May 9, 1945, which had indicated a very poor nutritional condition of the population.

PROCEDURE

A representative sample of the population which included all age groups, both sexes, various occupations and economic levels was selected in the following manner. An organization meeting was held in the office of the Director of the City Health Service on May 29th to explain the general plan and purpose of the survey. Representatives of the ration distribution office, the volunteer women's organization, the domestic science schools and the public health service attended this meeting, and definite plans were developed for conducting the survey. It was agreed that a total of approximately 2500 people should be examined and that these should be divided on the basis of 50% from the poor, 40% from the middle, and 10% from the upper classes to be representative of the entire population. Experience with previous surveys had shown that approximately 250 persons could be examined in 1 day at 1 location, hence 5 poor areas of the city, 4 middle class areas, and 1 upper class area were chosen carefully to assure scattering of the sample throughout the city area. Each such area contained approximately 2000 people so that a valid sample was obtained.

A second conference was held on the following day in the ration distribution office to consolidate plans for obtaining complete information on the past and present food ration and all other available food. Information obtained during

previous surveys indicated that the food situation had been steadily deteriorating during the German occupation until the autumn of 1944 when it became acute due to a national railway strike and the resulting punishment policy that was inflicted by the oppressors (Stare, '45). It was hoped that quantitative estimates of actual food consumption during 3 progressive periods, October, 1944, February, 1945, and April, 1945, might be obtained in order to establish a valid sequence of data leading up to the present survey. This plan was accomplished through the efforts of the household science teachers of the city who questioned approximately 90 families, in proper proportion, from poor, middle and upper class communities with regard to all food consumed during those months. Arrangements were also made to obtain records of food distributed by the Central Kitchen Organization, the Inter-Church Organization, and the Red Cross.

Personnel in a representative sample of the hospitals, rest homes, old folks homes, orphanages, prisons, and asylums, as well as in all 9 emergency hunger hospitals were also examined.

The actual examination of the general population was conducted at a prearranged time and place in each of the 10 sections of the city. Here the volunteer women's organizations were found to be invaluable not only in contacting the chosen families and seeing that they reported for examination at the proper time, but also in assisting with the recording of data and in maintaining the even flow of patients through the examining rooms. At the start of the examination each person was presented with a record card that contained the necessary blank spaces for entry of personal data, subjective complaints, height, weight, age, physical examination data, and laboratory determinations to be recorded as the bearer progressed through the clinic. In about 10% of the cases selected at random, complete dietary histories of food consumed during the past 24 hours and finger blood samples for hemoglobin and serum protein determinations by the gravity gradient method were obtained. In addition, approximately 100 venous blood

samples were drawn at random from adult personnel and were analyzed by the mobile laboratory for plasma vitamin A, plasma carotene, ascorbic acid, hematocrit, white cell volume, hemoglobin, and total serum protein.

RESULTS

In the rapid and detailed surveys of Rotterdam which were conducted on May 9, 1945, and May 29 to June 8, respectively, nutritional conditions were found to be very severe with large numbers of the population exhibiting evidence of insufficient food consumption. Eighty-six per cent of 154 subjects examined in the first survey were classified as thin, very thin, or emaciated with an estimated adult weight loss of 30 pounds. Hunger edema was found frequently, being recorded in nearly 20% of those examined. Children between the ages of 1 to 12 years and adults over 60 years of age were in the poorest nutritional condition, with the aged most severely affected. It was estimated from the survey data as well as by public health and other medical personnel that there were in the city 40,000 cases showing some degree of famine edema. Many deaths due to starvation were stated to have occurred, the total number being unavailable at the time of the studies. However, the death rate during the period January to April, 1945, had increased more than 100% over that of the previous year (table 1). Evidences of specific vitamin deficiencies were rarely noted. The average daily food consumption at the time of the early survey was approximately 1000 Cal., with a range of 500–1500 Cal. The dietary consisted mainly of coarse bread, potatoes, sugar beets, other available fresh vegetables, and a very limited amount of cereals, dairy products and meat.

In the detailed survey conducted from May 29 to June 8, 1945, a total of 2660 people, selected to be representative of the general population, were examined. In addition, 185 persons were examined in 20 institutions chosen to be representative of the hospitals, asylums, orphanages and old people's homes. Most of the hospitalized hunger patients were con-

centrated in emergency hunger hospitals, and hardly without exception these patients showed severe cachexia. Patients in other hospitals did not present a major problem in underfeeding.

TABLE 1

General mortality — Rotterdam (January–April).

	1944	1945	INCREASE (%)
Infants (0–1 yr.)	126	319	153
Children (1–5 yrs.)	78	173	122
Adults (5–65 yrs.)	910	2314	154
Males	495	1715	246
Females	415	599	44
Adults (65 + yrs.)	1179	3148	167
Males	537	2019	276
Females	642	1129	76
Total	2293	5952	159
Males	1159	4032	248
Females	1134	1920	69
	1944	1945	INCREASE (%)
Death rate (per 1000) — Rotterdam	11.5	29.8	159
Death rate (per 1000) — Boston	12.9	13.0	1

The grading in physical appearance of the people examined on May 9th as compared with those examined in the later survey is shown in table 2. An improvement in physical appearance, apparently took place in the interval between the 2 surveys. The 2 groups, however, were not strictly comparable in that the early survey was conducted in a relatively poor area of the city while the latter more detailed study included representative groups of all economic means. In grading the physical appearance scores in the latter survey approximately 40% of the poor people were judged normal, 50% of the middle class and 80% of the upper class. The estimated weight loss averaged 25 pounds in the groups from 19 to 59 years of age and 40 pounds in those over 60 years, with females showing slightly more loss than males.

The symptoms of weakness, fatigue, diarrhea, muscle pain and paresthesia were prevalent in all groups, especially in the aged. Amenorrhea was frequently reported.

Papillary atrophy, pallor, and follicular hyperkeratosis were the commonest clinical findings of nutritional interest occurring in 20 to 80% of the people and to a greater degree in those from the poor districts. Edema was present in about 3% of the people from the poor districts in the 19- to 59-year age group, and in nearly 12% of those over 60 years of age. This shows a considerable decrease in the incidence of

TABLE 2

General physical appearance — Rotterdam (May 9, 1945; May 29–June 8, 1945).

	TOTAL NO. EXAMINED		NORMAL	FAIR	POOR	SEVERE
			%	%	%	%
Adults	May	154	14	36	44	6
	June	1511	48	46	6	0
Children	May	118	10	31	31	28
	June	1149	22	44	34	0
"Hunger patients"						
Adults	May	127	0	0	25	75
	June	242	0	6	76	18
Children	May	none				
	June	380	0	5	62	33

edema as compared to the 20% average observed in the earlier survey..

Hemoglobin and total serum protein values, summarized in table 3, varied from 11 to 14 gm per 100 ml and 6 to 7.6 gm per 100 ml, respectively, with little difference between economic groups. Plasma volume was determined in the emergency hunger hospitals and was found to be 20 to 30% below normal in hunger patients. Average hemoglobin and total serum protein values in this group were only slightly below the general population averages although much greater variation was noted.

In addition to the finger blood determinations, approximately 100 venous samples were obtained at random from adult males and females and were analyzed in the mobile laboratory with the following results: hematocrit 41.4 volume %; white cell volume 1.0 volume %; hemoglobin 13.4 gm per 100 ml; serum protein 7.5 gm per 100 ml; plasma vitamin A 108 I.U. per 100 ml; plasma carotene 181 I.U. per 100 ml; and whole blood ascorbic acid 0.37 mg per 100 ml. Values obtained from different economic groups showed no significant variation.

TABLE 3

Average hemoglobin and serum protein values — Rotterdam (May 29–June 8, 1945)

AGE	NUMBER OF DETERMINATIONS	HEMOGLOBIN	SERUM PROTEIN
years		*gm/100 ml*	*gm/100 ml*
	Males		
1–12	25	12.5	7.1
13–18	17	13.1	7.3
19–59	57	13.8	7.4
60 +	17	12.5	6.9
	Females		
1–12	24	12.2	7.0
13–18	11	12.5	7.5
19–59	77	12.2	7.4
60 +	8	12.0	7.5

The average daily consumption of nutrients by the 3 economic groups is presented in table 4 and represents nearly 100% of the official food ration at that time. It should be noted that the food intake increased from 1000 to 2000 Cal. in the 4-week period between the 2 surveys. Simultaneously the official ration rose from 340 to 2000 Cal. This was due largely to the rapid import and distribution of Allied food supplies. Food was distributed mainly through the Rotterdam Food Distribution Office with some additional quantities handled through the Central Kitchen, Inter-Church and Red Cross organizations.

Obviously the daily food consumption of approximately 2000 Cal. at the time of the detailed survey did not account for the clinical evidences of undernutrition found. Likewise, it would be inaccurate to ascribe the nutritional condition of the population to the daily consumption of 1000 Cal. at the time of the rapid survey 1 month earlier. In an effort to obtain at least a rough estimate of the successive dietary levels which were responsible for the conditions found in the 2 surveys, dietary histories of food consumption during 3 separate

TABLE 4

Average daily nutrient consumption — Rotterdam (May 9, 1945; May 29–June 8, 1945).

NUMBER QUESTIONED	CALORIES	PROTEIN	FAT	CHO	Ca	Fe	VIT. A	B_1	B_2	NIACIN	C
		gm	*gm*	*gm*	*gm*	*mg*	*I U.*	*mg*	*mg*	*mg*	*mg*
		1 poor class district: May 9, 1945									
27	1044	46	5	204	0.5	8	2600	0.9	0.9	13	164
		5 poor class districts: June 1–8, 1945									
152	1851	53	45	243	0.7	12	6440	1.2	1.3	9	85
		4 middle class districts: June 1–8, 1945									
77	1965	58	48	268	0.6	15	9840	1.4	1.4	12	100
		1 upper class district: June 1–8, 1945									
21	2360	74	77	345	1.0	17	12900	2.0	1.6	13	169

monthly periods were obtained by the household science teachers. October, 1944, February and April, 1945, were chosen upon the advice of city authorities to represent successively the period preceding extreme food shortage, the winter period of hunger, and the period of extreme starvation prior to liberation. Average food consumption, as shown in figure 1, decreased from about 1700 Cal. to 1400 Cal. from the fall of 1944 to the spring of 1945 and then fell abruptly to about 1000 Cal. at the time of liberation as estimated by the rapid survey on May 9, 1945.

Although all food consumption data obtained through dietary questioning are subject to considerable error it should be

noted that the values obtained parallel the actual published official rations for the same periods. In a test of accuracy held in Paris a few months previous to this work, the dietary questioning technique used in the surveys was found to yield results within 10% of actual consumption figures.

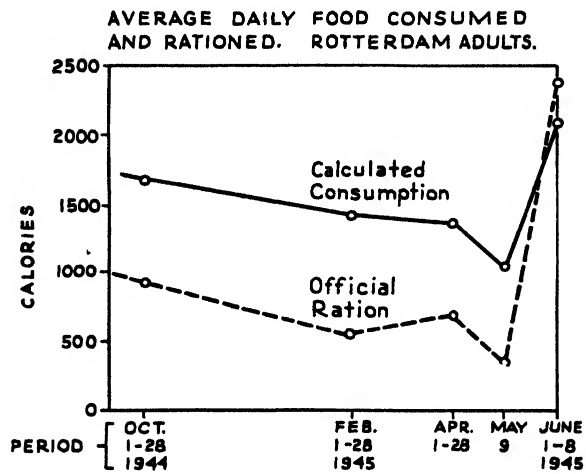


Figure 1

SUMMARY

Clinical, biochemical, and dietary data obtained in 2 nutritional surveys of Rotterdam, Holland, on May 9, 1945, and May 29 to June 8, 1945, are presented. A total of nearly 3000 people were examined.

In physical appearance 86% were classified as thin, very thin or emaciated in the early survey, and approximately 50% in the detailed survey 1 month later. Weight loss averaged about 25 pounds in the 19– to 59–year age group and 40 pounds in those over 60 years of age.

Symptoms of weakness, fatigue, diarrhea, muscle pain and paresthesia were prevalent, especially in the aged.

Papillary atrophy, pallor and follicular hyperkeratosis were common clinical findings.

Hunger edema was present in 20% of the people from poor class districts in the early survey, and in 3% and 12% of the

19- to 59-year age group and those over 60 years of age, respectively, in the later study.

Hemoglobin and total serum protein values averaged 12 to 14 gm per 100 ml and 7.0 to 7.5 gm per 100 ml, respectively.

Estimates of daily food consumption in 3 successive periods prior to the surveys were 1700, 1400, and 1400 Cal., respectively. The survey of May 9, 1945, indicated a further reduction in food consumption to an average intake of about 1000 Cal. The dietary consisted mainly of bread, potatoes, sugar beets and other vegetables. Considerable quantities of Allied food supplies were being distributed at the time of the second survey and the food consumption then averaged 2000 Cal. per day.

ACKNOWLEDGMENT

The authors wish to acknowledge their deep appreciation, especially to Lieut. Willem Nolen, Dr. von Andel, Dr. C. Den Hartog, and associates who contributed all of their time and energy to the successful prosecution of the surveys, and to the participating groups in Rotterdam for their wholehearted cooperation.

The leadership of Major Marvin B. Corlette, M.C., during the period of rapid surveys prior to May 7, 1945, is gratefully acknowledged.

LITERATURE CITED

BURGER, G. C. E., H. R. SANDSTEAD AND SIR JACK DRUMMOND 1945 Starvation in Western Holland. Lancet, Sept. 1, p. 282.

JOHNSON, R. E. 1945 Laboratory Manual of Field Methods. Harvard Fatigue Laboratory, Boston, Massachusetts.

LOWRY, O. H., AND T. H. HUNTER 1945 The determination of serum protein concentration with a gravity gradient tube. J. Biol. Chem., *159:* 465.

STARE, F. J. 1945 Nutritional conditions in Holland. Nutrition Revs., *3:* 225.

THE INFLUENCE OF AUTOCLAVING SOYBEAN OIL MEAL ON THE DIGESTIBILITY OF THE PROTEINS[1]

ROBERT JOHN EVANS, JAMES McGINNIS AND J. L. ST. JOHN
Divisions of Chemistry and Poultry Husbandry, Washington Agricultural Experiment Stations, Pullman

(Received for publication November 12, 1946)

That heating influences the nutritive value of soybean proteins has been recognized for several years. Evans and McGinnis ('46) reviewed the literature on this problem. They also presented additional data showing that moderate heating of raw soybean oil meal[2] increased its nutritive value for the growing chick. However, when such a meal was autoclaved at 130°C. for 30 or 60 minutes, the nutritive value was decreased. Ham and Sandstedt ('44) and Bowman ('44) have reported the presence of a trypsin inhibitor in soybean oil meal which is destroyed by heat. They believe that destruction of this enzyme inhibitor by heating accounts for the increased nutritive value of heated soybean oil meal. Evans ('46) studied the influence of autoclaving soybean oil meal on the liberation of amino groups from the soybean oil meal by different enzymes and enzyme combinations and found that when trypsin or trypsin and erepsin were used, a marked increase in digestibility resulted from autoclaving the soybean oil meal at 100°C. for 30 minutes. When treatment with

[1] Published as Scientific Paper no. 693, College of Agriculture and Agricultural Experiment Stations, Institute of Agricultural Sciences, State College of Washington, Pullman, Washington.

[2] The raw soybean oil meal used by Evans and McGinnis ('46) and that used in the present study was prepared by grinding solvent-extracted soybean flakes in a hammer mill followed by a thorough mixing.

pepsin preceded these digestions, no differences were observed. In all cases, autoclaving at 130° C. for 60 minutes very markedly decreased the liberation of amino groups by any of the enzyme combinations.

It was felt desirable to study further the digestibility of soybean oil meal proteins as influenced by autoclaving. The criteria chosen were digestibility of the proteins and sulfur compounds. The sulfur digestibility was considered to be especially important since Almquist, Mecchi, Kratzer and Grau ('42) believed methionine to be the principal growth limiting deficiency in soybean proteins. It is the purpose of the present paper to report the influence of the autoclaving of soybean oil meal upon the digestibility of total protein, the sulfur-containing proteins, the cystine-containing proteins, and the methionine-containing proteins as determined by the quantity of protein, sulfur, cystine and methionine remaining in the undigested protein fraction. Three digestion procedures were used: (1) the chick, (2) *in vitro* pepsin, trypsin, and erepsin, and (3) *in vitro* trypsin, and erepsin. Using chick data as a standard, comparisons were made with the 2 *in vitro* digestion procedures in an attempt to determine which most nearly approached the chick digestion. Two comparisons were made: (1) of the actual percentage of undigested protein and (2) of the effect of autoclaving on the raw protein. A graph in which the percentage of undigested protein is plotted against the autoclaving temperature best shows this second comparison. If the graph of one of the *in vitro* enzyme digestions parallels that for the chick digestion the 2 may be said to be affected similarly by the autoclaving treatment of the soybean oil meal. Such a parallel between curves is most readily shown by means of a coefficient of correlation calculated from the data presented in table 1 between the protein of the 6 soybean oil meals not digested by the chicks and that not digested by either of the *in vitro* enzyme combinations. For each coefficient of correlation 6 values (1 from each soybean oil meal) for the enzyme digests were correlated with 6 group values for chick digestion.

EXPERIMENTAL

This experiment is a continuation of the work reported earlier by Evans and McGinnis ('46). The data reported in the present paper were obtained from the 6 groups of 15 chicks each that received, in addition to the basal diet, 0.2% choline and were designated as groups 4 to 9 in the earlier study. Evans and McGinnis ('46) have described the methods of preparing the soybean oil meals, the diets fed, care of chicks, and collection of the droppings for chemical analyses. Each group of chicks was considered as a unit in the balance studies. All nitrogen determinations were made by the Kjeldahl-Gunning-Arnold method (A.O.A.C., '45). Uric acid nitrogen was determined by the method of St. John and Johnson ('31). Total sulfur was determined as described by Evans and St. John ('44), and organic sulfur as described by Evans and Greaves ('37). Cystine and methionine were determined by the differential oxidation procedure (Evans, '45b).

The nitrogen precipitated from the droppings by trichloroacetic acid minus the uric acid nitrogen in the droppings was considered to be the nitrogen not digested by the chick. It was necessary to subtract the uric acid nitrogen from the trichloroacetic acid precipitated nitrogen because uric acid is insoluble in acid solutions. Undigested organic sulfur, cystine and methionine were considered to be the amounts of these materials in the droppings precipitated by or insoluble in a 5% solution of trichloroacetic acid. To determine the trichloroacetic acid precipitated fraction 2.0 gm of the droppings were suspended by stirring with 50 ml of 5% trichloroacetic acid. The insoluble material was filtered on a no. 42 Whatman Filter Paper, washed 3 times with hot water containing a little trichloroacetic acid and the desired determination made on the residue. From the nitrogen intake of the group from the total diet, during the 1 week duration of the experiment, and the amount of undigested nitrogen in the droppings from the group, the per cent of nitrogen in the diet that was not digested was calculated. Similar calculations

were made to determine the per cent of the organic sulfur, cystine and methionine in the undigested protein fraction.

In vitro enzyme digestions were carried out as described by Evans ('46) using pepsin, trypsin and erepsin as 1 enzyme combination, and trypsin and erepsin as the other. Trichloroacetic acid precipitations were made of these digests as described by Evans ('46). The per cent glutelin in the different heat-treated soybean oil meals was determined by the method of Lund and Sandstrom ('43).

RESULTS AND DISCUSSION

The influence of the autoclaving of soybean oil meal on the digestibility of protein is shown in table 1. In calculating the percentage of protein in the soybean oil meal not digested by the chick, the assumption was made that all of the undigested protein was from the soybean oil meal and that protein from all other sources was completely digested. The validity of this assumption may be questioned, but it is reasonable to assume that the same percentage of protein from the other sources (5% brewer's yeast and 5% gelatin) was digested by all groups. The same method of calculation was used for organic sulfur, cystine sulfur and methionine sulfur.

Autoclaving raw soybean oil meal at 100–130°C. for 30 minutes increased digestion of the proteins by the chick and by trypsin and erepsin *in vitro,* but did not increase protein digestion by pepsin, trypsin and erepsin *in vitro.* Autoclaving at 130°C. for 60 minutes, in all cases, decreased protein digestion from that obtained after less severe autoclaving. A comparison of the per cent total protein not digested by the chick and that not digested by *in vitro* enzyme combinations shows that the chick digestion in magnitude more nearly approached that of pepsin, trypsin, and erepsin *in vitro* than that of trypsin, and erepsin. The chick digested the protein less completely than pepsin, trypsin, and erepsin *in vitro* and more completely than trypsin and erepsin *in vitro.* A highly significant coefficient of correlation of + 0.998 between the soybean oil meal protein not digested by the chick and that

TABLE 1

The influence of autoclaving soybean meal on the digestibility of the proteins.

	Heat treatment of soybean oil meal					
Time, *minutes*	none	30	30	30	30	60
Temperature, °*C.*	none	100	110	120	130	130
	Protein digestibility data with the chick[1]					
Nitrogen intake from soybean oil meal, *gm*	22.6	45.2	38.8	43.1	31.1	21.8
Nitrogen ppt. from droppings by trichloroacetic acid, *gm*	16.9	30.0	25.7	25.4	20.5	14.8
Uric acid nitrogen, *gm*	11.7	24.8	21.7	21.2	16.9	11.3
Undigested nitrogen,[2] *gm*	5.2	5.2	4.0	4.2	3.6	3.5
Undigested protein in soybean oil meal,[3] %	23.0	11.5	10.3	9.7	11.6	16.1
	Soybean oil meal protein not digested by in vitro enzyme digestion					
Pepsin, trypsin, erepsin, %	10.9	6.7	7.8	10.2	11.0	20.7
Trypsin, erepsin, %	34.9	15.9	12.5	12.4	15.4	23.1
	Organic sulfur digestibility data with the chick					
Organic sulfur intake from soybean oil meal, *gm*	1.16	2.31	1.98	2.20	1.59	1.11
Organic sulfur ppt. from droppings by trichloroacetic acid, *gm*	0.57	0.74	0.68	0.65	0.54	0.49
Undigested organic sulfur in soybean oil meal,[3] %	49.1	32.0	34.3	29.5	34.0	44.1
	Organic sulfur in soybean oil meal protein not digested by in vitro enzyme digestion					
Pepsin, trypsin, erepsin, %	19.4	24.4	23.6	31.3	29.2	40.8
Trypsin, erepsin, %	45.6	28.1	26.0	27.5	29.4	38.2
	Cystine digestibility data with the chick					
Cystine sulfur intake from soybean oil meal, *gm*	0.71	1.42	1.22	1.35	0.98	0.68
Cystine sulfur ppt. from droppings by trichloroacetic acid, *gm*	0.46	0.64	0.56	0.58	0.49	0.39
Undigested cystine sulfur in soybean oil meal,[3] %	64.8	45.1	45.9	43.0	50.0	57.4
	Cystine in the soybean oil meal protein not digested by in vitro enzyme digestion					
Pepsin, trypsin, erepsin, %	21.2	29.9	28.1	35.1	40.7	49.8
Trypsin, erepsin, %	50.6	35.9	33.8	30.3	32.0	41.6
	Methionine digestibility data with the chick					
Methionine sulfur intake from soybean oil meal, *gm*	0.45	0.89	0.76	0.85	0.61	0.43
Methionine sulfur ppt. from droppings by trichloroacetic acid, *gm*	0.10	0.06	0.08	0.05	0.04	0.11
Undigested methionine sulfur in soybean oil meal,[3] %	22.2	6.7	10.5	5.9	6.6	25.6
	Methionine in the soybean oil meal protein not digested by in vitro enzyme digestion					
Pepsin, trypsin, erepsin, %	16.4	15.8	16.4	25.3	16.4	26.7
Trypsin, erepsin, %	37.7	15.8	13.7	23.3	25.3	32.9

[1] Each group contained 15 chicks.
[2] Undigested nitrogen was the nitrogen precipitated by trichloroacetic acid minus the uric acid nitrogen.
[3] These values are calculated by making the assumption that all of the protein (nitrogen) other than that from the soybean oil meal was completely digested and hence that all of the undigested protein was from the soybean oil meal.

not digested by trypsin and erepsin *in vitro* and a non-significant correlation + 0.364 between the protein not digested by the chick and that not digested by pepsin, trypsin, and erepsin *in vitro* were obtained. These data indicate that, although in most cases the percentages of protein not digested by the chick were closer to the pepsin, trypsin, and erepsin values, because of the raw meal if the values are plotted against heat treatment the chick curve more nearly approaches the trypsin and erepsin curve. These data are interesting in view of the statement by Schoening ('39): "Strictly speaking, there is no true gastric digestion in the fowl such as is common to mammals." A poor pepsin activity in the chicken might well be expected. It appears probable, however, that some pepsin activity or other activity may be present in the chick because of greater digestion by the chick than by the enzymes trypsin and erepsin alone.

Data are presented in table 1 on the influence of autoclaving soybean oil meal on the per cent of the organic sulfur remaining in the undigested protein fraction. Autoclaving the raw meal for 30 minutes at 100–130°C. increased digestibility of the sulfur containing proteins for the chick and for trypsin and erepsin *in vitro*, but decreased it for pepsin, trypsin, and erepsin *in vitro*. Autoclaving for 60 minutes at 130°C. in all cases gave a soybean oil meal in which more of the sulfur was present in the undigested protein than when the meal was autoclaved less drastically. *In vitro* enzyme digestion with trypsin and erepsin or with pepsin, trypsin, and erepsin (1 exception) gave more complete digestion of the sulfur compounds than did digestion by the chick. In magnitude the *in vitro* trypsin and erepsin digestion more nearly corresponded to the chick digestion. A coefficient of correlation between organic sulfur not digested by the chick and that not digested by *in vitro* trypsin and erepsin digestion of + 0.959 was obtained. A value of 0.917 was necessary for the results to be highly significant. The correlation coefficient between *in vitro* pepsin, trypsin, and erepsin digestion and digestion by the chick was + 0.031. Autoclaving soybean oil meal for

30 minutes at 100°C. decreased the amount of organic sulfur in soybean oil meal not digested by the chick from 49% to 32%. This is of interest since Johnson, Parsons and Steenbock ('39) observed no differences in digestibility of sulfur compounds in raw and cooked soybean oil meals, as determined by sulfur balance determinations with adult rats. Evans and McGinnis ('46) observed an increase in retention of organic sulfur as a result of autoclaving raw soybean oil meal. It appears, therefore, that the adult rat may be able to digest the sulfur containing proteins of raw soybean oil meal to a greater extent than can the growing chick. This might be due to differences between the type of protein digestion that prevails in the growing chick as compared to the adult rat. Pepsin in the adult rat might digest sulfur-containing proteins that would otherwise not be acted upon. This could well be explained on the basis of a digestion by pepsin of the trypsin inhibitor present in soybean oil meal which was shown by Kunitz ('45) to be a protein. Ham, Sandstedt and Mussehl ('45) demonstrated that the trypsin inhibitor when added to chick diets retarded growth of the chicks. McGinnis and Menzies ('46) showed that *in vitro* papain digestion improved the nutritive value of raw soybean flakes as effectively as did autoclaving for chicks. Carver, McGinnis, McClary and Evans ('46) observed equally good egg production of hens receiving raw and those receiving heat-treated soybean oil meal as the protein concentrate.

Data concerning the influence of autoclaving soybean oil meal on the per cent of the total cystine and methionine in the undigested protein fraction are presented in table 1. Autoclaving the raw soybean oil meal at 100–130°C. for 30 minutes increased digestibility of the cystine and methionine-containing proteins for the chick and for trypsin ard erepsin *in vitro*. Digestibility of the cystine-containing proteins for pepsin, trypsin and erepsin *in vitro* was decreased by autoclaving, but the digestibility of the methionine-containing proteins by this enzyme combination was not affected except at higher autoclaving temperatures which in 2 cases decreased digestibility.

A highly significant coefficient of correlation of + 0.936 between cystine sulfur of soybean oil meal not digested by the chick and that not digested by trypsin and erepsin *in vitro* was obtained. Low correlation (+ 0.108) was observed between the chick digestion of cystine-containing proteins and pepsin, trypsin and erepsin digestion *in vitro*. As with organic sulfur, the *in vitro* digestion of cystine-containing proteins by trypsin and erepsin or pepsin, trypsin, and erepsin was more complete than was digestion by the chick. In magnitude the *in vitro* trypsin and erepsin digestion most nearly approached the chick digestion. Autoclaving the raw soybean oil meal at 130°C. for 60 minutes more than doubled the cystine in this protein fraction and nearly doubled the protein. Also of interest was the observation that the cystine-containing proteins of the soybean oil meals which were autoclaved at 120°C. or higher were more completely digested *in vitro* by trypsin and erepsin than by pepsin, trypsin and erepsin, though trypsin and erepsin digested the uncooked cystine-containing proteins or those cooked at lower temperatures to a lesser extent than the pepsin, trypsin, and erepsin combination. No explanation for these results can be offered at this time although it might be in some way tied up with heat denaturation and its effect on the —SH and —S—S— groups. It is recognized that heat denaturation in some way changes the activity of these groups (Anson, '45).

The chick digested a larger proportion of the ingested methionine present in any of the soybean oil meals than was digested *in vitro* by trypsin and erepsin, and digested more from all of the meals except the raw one than was digested *in vitro* by pepsin, trypsin and erepsin (table 1). These values for the chick are of especial interest because of the low percentage of methionine in the undigested protein fraction except for the raw soybean oil meal and the one that was autoclaved for 60 minutes at 130°C. The *in vitro* pepsin, trypsin, and erepsin values more nearly agree with the chick values in magnitude. A coefficient of correlation of + 0.766 was obtained between the per cent of soybean oil meal methi-

onine not digested by the chick and that not digested by trypsin and erepsin. One of 0.811 was required for significance. Neither the cystine nor methionine undigested by pepsin, trypsin, and erepsin were correlated with those undigested by the chick. It appears evident from the data presented in table 1 that soybean oil meal contains a protein high in cystine content which is poorly digested by the chick or by the *in vitro* enzyme combinations used. On the other hand, the percentage of the total methionine not digested by the chick or by *in vitro* enzyme digestion is in the same magnitude as the percentage of the total protein not digested in these ways

TABLE 2

The influence of autoclaving soybean oil meal on the nutritive value for chicks, per cent glutelins, and amino groups liberated by in vitro enzyme digestion.

HEAT TREATMENT OF SOYBEAN OIL MEAL		GAIN IN WEIGHT OF CHICKS TO 4 WEEKS [1]	PROTEIN EFFICIENCY [1]	GLUTELINS	AMINO GROUPS LIBERATED BY IN VITRO ENZYME DIGESTION	
Time	Temp.				Pepsin, trypsin, erepsin [2]	Trypsin, erepsin [2]
min.	*°C.*	*gm*	*gain/protein*	%	%	%
None		76	1.24	25.4	35	22
30	100	174	1.81	49.0	36	31
30	110	156	1.76	56.7	36	32
30	120	162	1.66	36.5	37	33
30	130	120	1.57	18.8	33	28
60	130	69	1.09	7.0	29	24

[1] These values are taken from the paper by Evans and McGinnis ('46).
[2] These values are taken from the paper by Evans ('46).

(table 1). Melnick, Oser and Weiss ('46) suggest that the difference in nutritive value of raw and autoclaved soybean oil meals is due to a difference in rate of liberation of methionine. Data in table 1 indicate that the amount of methionine remaining in the protein fraction not digested by the chick is greater for raw than for properly autoclaved soybean oil meal.

In summarizing the work to date from this laboratory with regard to the influence of autoclaving soybean oil meal on the nutritive value and digestibility of the protein, the data presented in table 2 are of interest. A comparison of the

protein efficiency, as measured by the grams gain in weight of growing chicks during a 4-week period divided by the grams of protein consumed during this period, and the amino groups liberated by *in vitro* trypsin and erepsin digestion of the soybean oil meal showed a coefficient of correlation of + 0.903. One of 0.811 was required for significance. A coefficient of correlation of + 0.769 which was not significant was obtained between protein efficiency and amino groups liberated by *in vitro* pepsin, trypsin, and erepsin digestion. These values indicate that autoclaving affects soybean oil meal in the same way for liberation of amino groups by trypsin and erepsin *in vitro* and for efficiency of chick protein utilization. It would appear from these results and those presented in table 1 that the major part of the digestive activity was characteristic of a trypsin and erepsin system. This may at least partially explain why attempts by various workers in the past have not shown a relation between *in vitro* pepsin digestion and true digestibility or biological value for soybean oil meals. Evans ('45a) observed no relation between the pepsin digestible sulfur of commercial soybean oil meals and their protein nutritive value for chicks.

The per cent glutelin in 10 commercial soybean oil meals was shown by Evans and St. John ('45) to have a highly significant correlation with the protein nutritive values as measured by the gross protein value determined by the procedure of Heiman, Carver and Cook ('39) when no uncooked soybean oil meal was included in the calculation. The values for per cent of protein measured as glutelin in the soybean oil meals studied in this experiment are presented in table 2. The highest glutelin value was in the meal autoclaved for 30 minutes at 110°C. Autoclaving increased the per cent protein measured as glutelin (soluble in 0.2% KOH solution but insoluble in water, 5.0% KCl solution or 70% alcohol) over that in the raw soybean oil meals except for the meals autoclaved at 130°C. for 30 or 60 minutes which contained less protein determined as glutelin than the raw meal. Excluding the raw soybean oil meal from the calculations, a coefficient of

correlation of + 0.887 between per cent glutelin and protein efficiency was obtained. A value of 0.878 was required for significance. Including the raw soybean oil meal in the calculations, a coefficient of correlation of + 0.854 was obtained. One of 0.811 was required for significance.

SUMMARY

Chicks were raised to 4 weeks of age on diets supplemented with raw soybean oil meals which had received the following treatments: none, autoclaved for 30 minutes at 100, 110, 120, 130°C. or autoclaved for 60 minutes at 130°C. The percentages of total protein, organic sulfur, cystine and methionine in the soybean oil meals not digested by the chick, by pepsin, trypsin and erepsin *in vitro,* or by trypsin and erepsin *in vitro* were determined. The soybean oil meals which had been autoclaved at temperatures between 100°C. and 120°C. for 30 minutes were more completely digested by the chick or by trypsin and erepsin *in vitro* than the raw meal or the meals which had been autoclaved at 130°C. using undigested total protein, or sulfur, cystine, or methionine in the undigested protein as criteria. Raw soybean oil meal was in most cases more readily digested by pepsin, trypsin and erepsin *in vitro* than the autoclaved meals. Significant correlations were obtained between *in vitro* trypsin and erepsin digestion, and chick digestion of these soybean oil meals for undigested protein, organic sulfur and cystine. No significant correlations between chick digestion and *in vitro* pepsin, trypsin and erepsin digestion values were obtained. Of especial interest is the relatively high percentage of cystine in the soybean oil meals that was not digested by the chick or by either of the *in vitro* enzyme digestions made.

LITERATURE CITED

ALMQUIST, H. J., E. MECCHI, F. H. KRATZER AND C. R. GRAU 1942 Soybean protein as a source of amino acids for the chick. J. Nutrition, *24:* 385.

ANSON, M. L. 1945 Protein denaturation and the properties of protein groups. Advances in Protein Chem., *2:* 361.

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1945 Official Methods of Analysis, par. 2.26, p. 27.

BOWMAN, D. E. 1944 Fractions derived from soybeans and navy beans which retard tryptic digestion of casein. Proc. Soc. Exp. Biol. Med., *57:* 139.

CARVER, J. S., J. MCGINNIS, C. F. MCCLARY AND R. J. EVANS 1946 The utilization of raw and heat-treated soybean oil meal for egg production and hatchability. Poultry Sci., *25:* 399.

EVANS, R. J. 1945a The sulfur content of the pepsin digestible fraction of protein concentrates. Arch. Biochem., *7:* 33.

——— 1945b Determination of the cystine and methionine content of plant and animal material by a differential oxidation procedure. Arch. Biochem., *7:* 439.

——— 1946 Hydrolysis of soybean oil meal proteins by some proteolytic enzymes. Arch. Biochem., *11:* 15.

EVANS, R. J., AND J. E. GREAVES 1937 Some factors influencing the sulfur content of alfalfa. Proc. Utah Acad. Sci., *14:* 17.

EVANS, R. J., AND J. MCGINNIS 1946 The influence of autoclaving soybean oil meal on the availability of cystine and methionine for the chick. J. Nutrition, *31:* 449.

EVANS, R. J., AND J. L. ST. JOHN 1944 Determination of total sulfur in feeds. Modified nitric and perchloric acid digestion procedure. Ind. Eng. Chem., Anal. Ed., *16:* 630.

——— 1945 Estimation of the relative nutritive value of vegetable proteins by 2 chemical methods. J. Nutrition, *30:* 209.

HAM, W. E., AND R. M. SANDSTEDT 1944 A proteolytic inhibiting substance in the extract from unheated soybean meal. J. Biol. Chem., *154:* 505.

HAM, W. E., R. M. SANDSTEDT AND F. E. MUSSEHL 1945 The proteolytic inhibiting substance in the extract from unheated soybean oil meal and its effect upon growth in chicks. J. Biol. Chem., *161:* 635.

HEIMAN, V., J. S. CARVER AND J. W. COOK 1939 A method for determining the gross value of protein concentrates. Poultry Sci., *18:* 464.

JOHNSON, L. M., H. T. PARSONS AND H. STEENBOCK 1939 Effect of heat and solvents on the nutritive value of soybean protein. J. Nutrition, *18:* 423.

KUNITZ, M. 1945 Crystallization of a trypsin inhibitor from soybean. Science, *101:* 668.

LUND, A. P., AND W. M. SANDSTROM 1943 The proteins of various tree seeds. J. Agr. Research, *66:* 349.

MCGINNIS, J., AND V. H. MENZIES 1946 Effect of in vitro enzymatic digestion of raw soybean flakes on chick growth. Poultry Sci., *25:* 538.

MELNICK, D., B. L. OSER AND S. WEISS 1946 Rate of enzymic digestion of proteins as a factor in nutrition. Science, *103:* 326.

SCHOENING, H. W. 1939 The digestive processes in domestic animals. Food and Life, Yearbook of Agric., U. S. Dept. of Agric., p. 425.

ST. JOHN, J. L., AND O. JOHNSON 1931 The determination of uric acid in the study of avian nutrition. J. Biol. Chem., *92:* 41.

NUTRITIVE VALUE OF KERATIN

III. EFFECT OF SOURCE, PARTICLE SIZE, AND METHOD OF GRINDING [1]

G. W. NEWELL AND C. A. ELVEHJEM
Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

(Received for publication December 30, 1946)

INTRODUCTION

Keratin is generally defined as a protein insoluble in dilute acid or alkali, and resistant to digestion by the common proteolytic enzymes. Meunier et al. ('27) however, showed that when wool is digested with alkali at pH 9–10, it becomes partially susceptible to the proteolytic action of pancreatin, and Routh and Lewis ('38) demonstrated an even greater digestion of wool by trypsin and pepsin after grinding in a ball mill.

Recently, reports have come from several laboratories concerning the nutritional value of powdered keratins. Routh ('42) used powdered wool, and later ('42a) powdered chicken feathers as the only source of protein in purified rations for rats. Growth response, although poor, was most marked when amino acid supplements of lysine, histidine, methionine and

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from Wilson and Co., Chicago, Ill.

We are indebted to the Wilson Laboratories, Chicago, Ill., for the liver products; to Wilson and Company, Chicago, Ill., for some of the keratin samples; to Merck and Company, Rahway, N. J., for the synthetic vitamins; and to the Abbott Laboratories, North Chicago, Ill., for the supply of Haliver Oil.

tryptophane were included in the rations. Wagner and Elvehjem ('43) found that powdered hog hoofs could be substituted for meat-scrap or fishmeal supplements in practical poultry rations. In the nutritional experiments cited the keratins were prepared by grinding in a ball mill for prolonged periods of time.

In this paper we wish to describe the growth of rats and chicks fed purified diets in which the protein was, in whole, or in part, keratin prepared by different methods. The effect of amino acid supplements on the growth of rats receiving certain of these keratin preparations is also presented.

EXPERIMENTAL

Two-day old chicks obtained from commercial hatcheries, 6 per group, were kept on raised wire screens in electrically heated cages. Weanling male rats, of the Sprague-Dawley strain, weighing 40 to 50 gm were kept in individual raised wire cages. Rations and water were fed *ad libitum* throughout the 4-week experimental period. The composition of the rations used is given in tables 1 and 3. In all cases the level of protein was varied at the expense of sucrose.

Keratins used in this study were hog hoofs, summer hog hair, and chicken feathers. These proteins were prepared by powdering in a ball mill for 3 to 7 days. Several samples of hoof keratins were subjected to different types of grinding procedures. Keratin-A was powdered in a ball mill, while keratin-B was ground in a hammer mill and then finally powdered in a Wiley mill. In both these methods considerable heat is developed during the grinding procedure. The third hoof sample, keratin-C, was prepared by a process which rapidly pulverizes the keratin to a fine powder without heating. All keratins were ground fine enough to pass an 80 mesh screen except in the experiment where particle size was studied. Special effort was made to determine which amino acids were necessary to supplement the powdered hoofs (18 and 24% levels) in order to obtain growth comparable to that of an equal level of casein.

RESULTS

Powdered keratin as a protein source in chick rations

The diets used for the chicks and the growth responses obtained are given in table 1. Group 1, which received casein and cartilage serves as the reference group. The growth obtained is representative of that produced by purified rations

TABLE 1

Effect of ball mill powdered keratins on growth and survival of chicks. (6 chicks per group.)

GROUP NO.	1	2	3	4	5	6	7	8	9	10	11
Casein [1]	18	18	10					10			18
Cartilage	10										
Powdered hog hair		10	18	24	30	30	30				
Powdered hog hoofs (keratin-A)								18	24	30	
Powdered chicken feathers											10
Powdered oat meal							5				
α-tocopherol mg/day/chick						1					
Sucrose	54	54	54	58	52	52	47	54	58	52	54
Average weight at 4 weeks	244	216	212	94	116	57	59	227	157	216	132
Encephalomalacia	—	+	+	+	+	—	—		—	—	—
Gizzard erosion											
Slight	3						4		1	1	3
Moderate	3	6	2	6		6	2		2	4	
Severe			4		3				3	1	3
Number dead at 4 weeks		1	2	1	3						

[1] All rations contained in addition to the above listed constituents: brewer's yeast, 5%; soybean oil, 5%; Salts IV, 4%; solubilized liver powder, 2%; cod liver oil, 2%; and vitamin supplements per kilo of ration as follows: thiamine hydrochloride, 3 mg; riboflavin, 3 mg; pyridoxine hydrochloride, 3 mg; calcium pantothenate, 15 mg; choline hydrochloride, 1.5 gm.

of this type. Powdered hog hair at levels of 24% or 30% of the ration produced poor growth (groups 4 and 5). Feathering of chicks fed this protein was only fair. High levels of powdered hog hair tended to cause the occurrence of nutritional encephalomalacia. When casein was combined with

hog hair in a ration at 18% and 10% (group 2), or at 10% and 18% (group 3), respectively, good growth was obtained. Encephalomalacia still occurred with these protein combinations but the incidence decreased as the percentage of powdered hair was reduced. Pappenheimer et al. ('39) were able to prevent the incidence of encephalomalacia in chicks fed purified rations by oral administration of small quantities of α-tocopherol. Two additional groups were fed 30% powdered hair; 1 group received oral supplements of 1 mg α-tocopherol per chick per day, and the other group was fed 5% powdered oats as a natural source of vitamin E. None of the chicks in these 2 groups exhibited any signs of encephalomalacia. The growth rate was only about half that obtained in group 5 but the hair used for groups 6 and 7 was ground for 6 days and this long grinding may have been responsible for the reduced growth. Group 11 included 10% chicken feathers and 18% casein as the protein source. Growth was only about half that obtained in the reference group even though the level of casein was not reduced. Feathering of chicks on this ration was very poor.

Poor growth resulted when powdered hoofs were fed at a 24% level (group 9) but when the level was increased to 30% (group 10), good growth was obtained. When 18% of powdered hoofs was combined with 10% of casein, the response obtained closely approximated the maximum growth of chicks fed the casein-cartilage reference ration. At all levels of hog hoofs used in these studies, good feathering was observed.

The gizzards of chicks fed the above-mentioned keratins were examined for erosion. Gizzards were graded according to the severity of the lesions as slight, moderate, or severe. It was not possible to use complete prevention of gizzard erosion as a criterion, since chicks under 6 weeks of age rarely have gizzards entirely free of lesions. Powdered hog hair consistently caused moderate to severe erosion of the gizzard. The majority of the fissures found were parallel to the normal folds of the linings. Chicken feathers were variable in their effect, but the severity of the lesions consistently fol-

lowed the poor feathering of the birds fed this protein. Gizzards of chicks fed powdered hog hoofs were comparatively free of erosion and compared favorably with the gizzards of chicks fed the reference ration.

During the course of these studies, a question arose as to what role the particle size of powdered keratin might play in the growth of chicks. It had been noticed that when keratins were powdered for long periods in a ball mill, the nutritive quality of the protein was materially decreased. An experiment was carried out in which powdered hog hoofs which passed screens with mesh sizes of 10, 20, 40, 60 and 80 were included in the ration at a level of 30% (table 2). One series

TABLE 2

Effect of different methods of grinding keratin on the growth of chicks[1] *(gm gained in 4 wks.; 6 chicks per group).*

MESH SIZE	10	20	40	60	80
Hog hoofs powdered in ball mill (keratin-A)		96	60	88	125
Hog hoofs ground successively in hammer and Wiley mills (keratin-B)	134	141	140	158	160

[1] Hog hoofs at 30% of ration constituted the sole protein.

of chicks received hoofs powdered in a ball mill (keratin-A) while the second series was fed powdered hoofs prepared in a hammer and a Wiley mill (keratin-B). That destructive action takes place in the ball mill is clearly demonstrated by the poor growth of chicks fed this product, regardless of the particle size. Hoofs powdered in the Wiley mill gave the best results when chicks were fed the material passing the 80 mesh screen. A gradual decrease in growth response was noted as the particle size was increased.

Powdered keratins as a source of protein for the growing rat

Results obtained when rats were fed rations in which keratin was used as the source of protein are given in table 3.

A group without any protein (R–I) and one given 18% casein (R–II) are included for reference.

When powdered hog hair was fed at a level of 18% of the ration (R–V) the rats showed a growth of only 6 gm in 4 weeks. The animals fed rations in which casein replaced one-third (R–IV) or one-half (R–III) of the hair showed greatly increased growth, although the response did not approach the rate obtained with animals which received 18% of casein.

TABLE 3

Effect of powdered keratins (ball milled) on growth of rats. (6 rats per group.)

RATION (R)[1]	I	II	III	IV	V	VI	VII	VIII	IX
Casein		18	9	6		9	6		
Powdered hog hair			9	12	18				
Powdered hog hoofs						9	12	18	24
Sucrose	89	71	71	71	71	71	71	71	71
Gm gained in 4 weeks	—15	139	109	101	6	133	123	55	60

[1] All diets contained in addition to the above listed constituents: Salts IV, 4%; corn oil, 5%; liver powder 1–20, 2%; and vitamin supplements per kilo of ration as follows: thiamine hydrochloride, 2 mg; riboflavin, 1 mg; pyridoxine hydrochloride, 3 mg; calcium pantothenate, 20 mg; choline chloride, 1 gm. Each rat received 1 drop of Haliver Oil per week.

When hoofs powdered in a ball mill were used as the sole source of protein at a level of 18% in the ration (R–VIII), a growth response of 55 gm was obtained in 4 weeks. The rate of growth is poor as compared with that obtained with 18% of casein but markedly better than that obtained with the same level of powdered hair. When the level of powdered hoofs was increased to 24% of the ration (R–IX) little improvement in growth resulted. With protein at 18% of the ration, substitution of one-third of the powdered hoofs with casein (R–VII) resulted in moderate growth, while a combination of 9% of hoofs and 9% of casein, (R–VI), produced growth approaching that obtained with 18% of casein.

Effect of method of preparation and of amino acid supplements on the growth of rats fed powdered hog hoofs

In these studies the rations were patterned after those described in table 3. Groups 1 and 2 receiving 18% and 24% of casein (lines 1 and 2, table 4), served as optimum reference groups.

TABLE 4

Growth response of rats to hog hoofs prepared by different methods. (3 rats per group.)

	RATION AND SUPPLEMENTS	GM GAINED IN 4 WEEKS ON: Keratin-A [1]	Keratin-C [2]
1	18% Casein	134	
2	24% Casein	143	
3	18% Hog hoofs (R VIII)	40	66
4	24% Hog hoofs (R IX)	42	112
5	30% Hog hoofs	112	130
6	40% Hog hoofs		140
7	R VIII plus 0.6% lysine, 0.1% tryptophane, 0.3% methionine, 0.6% cystine	27	
8	R VIII plus 0.6% lysine, 0.3% methionine, 0.6% cystine, 0.4% histidine	95	
9	R VIII plus 0.1% tryptophane, 0.3% methionine, 0.6% cystine, 0.4% histidine	91	
10	R VIII plus 0.6% lysine, 0.1% tryptophane, 0.3% methionine, 0.4% histidine	109	124
11	R IX plus amino acids as in 10		136
12	R VIII plus 0.6% lysine, 0.2% tryptophane, 0.3% methionine, 0.4% histidine		121
13	R IX plus amino acids as in 12		128
14	R VIII plus 0.6% lysine, 0.2% tryptophane, 0.6% methionine, 0.4% histidine		121
15	R IX plus amino acids as in 14		136
16	R IX plus 0.6% lysine, 0.2% tryptophane, 0.6% methionine, 0.4% histidine, 0.4% phenylalanine		137
17	R IX plus 0.6% lysine, 0.05% tryptophane, 0.3% methionine, 0.3% histidine, 0.3% phenylalanine		118

[1] Powered in a ball mill.

[2] Powdered with little or no heat production.

Data from the chick experiments indicate that there is a difference in the nutritive quality of keratins, dependent upon the method of preparation. As in the earlier experiments, growth of rats fed hoofs at an 18% level was poor. However, keratin-C (powdered with no heat production) gave a 55% better growth response than did keratin-A (powdered in a ball mill). When the level of hoofs was raised to 24% a more marked difference between the 2 keratin preparations could be noted. When keratin-C was included in the ration at 30%, a growth rate which approximated that obtained with 18% casein was obtained. Only rather moderate growth resulted when rats were fed keratin-A at this level. At 40% of the ration keratin-C produced growth comparable to that obtained with 24% casein. The hair coat of animals fed powdered hog hoofs was always heavy and glossy. At no time was there any indication of toxicity due to the feeding of high levels of keratin.

The necessity of feeding such high levels of keratin (30% to 40%) suggested that certain essential amino acids are probably present in limited amounts. This view is substantiated by the data of Routh ('42, '42a) which indicate that tryptophane, methionine, lysine and histidine are necessary supplements to diets where powdered wool or feathers supply the protein. In this laboratory amino acid supplements of 0.6% lysine, 0.1% tryptophane, 0.3% methionine and 0.6% cystine to ration R–VIII (line 7, table 4) produced a decrease in growth as compared with that obtained with 18% of keratin-A alone. Substitution of 0.4% histidine for the tryptophane produced a marked improvement of growth. The combination of tryptophane, methionine, cystine and histidine resulted in the same rate of growth. Elimination of cystine and use of the 4 amino acids, lysine, tryptophane, methionine and histidine, found most efficient by Routh in supplementing wool protein, gave the best growth. When keratin-C was increased to 24%, and the same amino acids used as before (line 11), young rats grew at a rate comparable to that produced by 18% of casein. No improvement was obtained with

R–VIII when the tryptophane (line 12) or the methionine (line 14) supplements were increased 100%. When these increased amino acid supplements were added to R–IX (lines 13 and 15), growth as good as that demonstrated in line 11 was obtained. Microbiological assay of keratin-C suggested that the amount of phenylalanine present was at a critical level if the protein were fed at 18% or even 24% of the ration. However, when 0.4% phenylalanine was added to R–IX, in addition to methionine, tryptophane, lysine and histidine (line 16) growth was the same as that obtained in line 11.

After examination of the amino acid values tabulated by Block and Bolling ('45) for horn keratins, calculations were made to determine the level of essential amino acids furnished by powdered hoofs when fed at 24% of the ration. These estimates suggested that if supplements of 0.6% lysine, 0.05% tryptophane, 0.3% methionine, 0.3% histidine and 0.3% phenylalanine were made to R–IX, a growth response comparable to that of 18% casein would be obtained. This expected response was not achieved since growth (line 17) was only slightly better than when unsupplemented R–IX was fed (line 4). These results may indicate that the portion of the amino acids supplied by powdered hoofs was only partially available.

DISCUSSION

The data presented indicate that rats and chicks are able to utilize powdered keratins although the origin and method of preparation markedly affect the biological value of these proteins. Hoofs gave the best growth response in both rats and chicks, while hair was definitely inferior. Feathers appeared to be the least suitable nutritional material of the 3 keratins tried.

The preparation of this type of protein also greatly influences its nutritional value. Data on the effect of particle size on the growth of chicks are presented and similar results, not tabulated, were obtained with rats. Not only must the product be finely divided but the method of bringing about

this breakdown is very important. Fineness of division appears necessary to speed enzymatic hydrolysis and prolonged grinding appears to produce nutritionally undesirable changes, possibly as a result of heating. Routh and Lewis ('38) have shown that inorganic sulfates not originally present in wool are produced by the prolonged ball mill grinding, and they suggest that these are oxidation products. Hog hoof keratin was the most intensively investigated but if methods are developed to reduce hair or feathers to sufficient fineness without producing undesirable changes, these keratins might become more valuable.

Ball mill grinding appears to be destructive from both mechanical and chemical standpoints. Briggs et al. ('42) have shown that erosion of the gizzard may occur when there is an insufficient supply of glycine, arginine, and cystine in the chick ration. The severity of gizzard erosion in chicks which received hair would imply that the amino acids present in adequate amounts in the original hair were not available to the chick. When fed powdered hair numerous chicks developed encephalomalacia which was cured to α-tocopherol. Why this condition developed on powdered hair and not on other keratins is not clear.

Substantial growth was obtained when ball milled hog hoofs were fed to rats and chicks at 30% of the ration. The specially ground hoofs at this level supported growth equal to that of 18% of casein. With supplements of lysine, tryptophane, methionine, and histidine, 18% of hog hoofs gave growth which also approximated that obtained with casein. Data, not tabulated here, have established that omission of any one of the 4 amino acids results in growth no better than that obtained without supplementation.

Because of the physical changes brought about when keratins are powdered, the practical application of new protein sources should not be overlooked. This work confirms the suggestions of Routh ('42) and Wagner and Elvehjem ('42), and indicates that careful preparation of keratinous material

offers a means whereby waste protein of high nutritive quality may be adapted for use in animal feeds.

SUMMARY

Finely powdered keratins have been studied as possible protein sources for growing rats and chicks. When chicks or rats were fed purified rations which contained 30–40% of powdered hoofs, substantial growth was obtained. High levels of powdered hog hair in chick rations allowed moderate growth but usually produced an encephalomalacia unless sources of vitamin E were present. Powdered chicken feathers allowed only poor growth.

In general, the rates of growth obtained with chicks and rats fed rations containing these keratins show a positive correlation with the degree of subdivision of the keratin. The use of a ball mill for grinding the keratin was not as effective a method as one in which heating was prevented. When the lower levels of powdered hoofs were supplemented with lysine, tryptophane, methionine, and histidine, good growth was obtained.

LITERATURE CITED

BLOCK, R. J., AND D. BOLLING 1945 The Amino Acid Composition of Proteins and Foods. 1st ed. Charles C. Thomas, Springfield.

BRIGGS, G. M., JR., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1942 The effect of added cystine in purified rations for the chick. J. Biol. Chem., *144:* 47.

MEUNIER, L., P. CHAMBARD AND H. COMTE 1927 Sur la digestion pancréatique de la laine. Compt. rendu des Societie de L'academie des Sciences, *184:* 1208.

PAPPENHEIMER, A. M., M. GOETTSCH AND E. JUNGHERR 1939 Nutritional encephalomalacia in chicks. Conn. Exp. Sta. (Storrs) Bull. 229, p. 121.

ROUTH, J. I. 1942 Nutritional studies on powdered wool. J. Nutrition, *23:* 125.

——— 1942a Nutritional studies on powdered chicken feathers. J. Nutrition, *24:* 399.

ROUTH, J. I., AND H. B. LEWIS 1938 The enzymatic digestion of wool. J. Biol. Chem., *124:* 725.

WAGNER, J. R., AND C. A. ELVEHJEM 1942 The nutritive value of keratins. I. Proc. Soc. Exp. Biol. Med., *51:* 394.

——— 1943 The nutritive value of keratins. II. Poultry Sci., *22:* 275.

A STUDY OF THE RELATION BETWEEN PROTEIN EFFICIENCY AND GAIN IN WEIGHT ON DIETS OF CONSTANT PROTEIN CONTENT[1]

D. M. HEGSTED AND JANE WORCESTER

Departments of Nutrition, Biological Chemistry, and Biostatistics, Harvard School of Public Health and Harvard Medical School, Boston

TEN FIGURES

(Received for publication January 17, 1947)

Osborne, Mendel and Ferry ('19) originally proposed the use of "grams gain per gram of protein eaten" (protein efficiency) as a measure of the nutritive value of proteins for growing rats. This index combined the 2 variables of food intake and gain in weight into a single figure and thus was thought to be a superior measure of nutritive value than the simple measurement of either variable alone, that is, gain in weight or food intake. In subsequent years, protein efficiency has gained wide acceptance as a measure of the nutritive value of proteins.

In the original paper, and subsequently, it was clearly shown that the level of the protein in the diet affects the value obtained for the protein efficiency. With increasing levels of protein, it reaches a maximum and then slowly declines. Osborne et al. proposed that the level of maximum efficiency be determined for each protein. In practice, however, most authors including the originators fed various proteins at arbitrary levels and compared the protein efficiency values thus obtained.

[1] Supported in part by a grant-in-aid from The American Meat Institute, Chicago, Ill.

Mitchell ('24, '42) has criticized this method on various scores, principally on the basis that the composition of the body gains may not be constant and that no credit is given to the protein for maintenance. He has observed (Mitchell, '24) that in general animals with larger food intakes tend to show the highest protein efficiency "although the correlation is not particularly close."

It is remarkable in view of the wide acceptance of the method as a measure of the nutritive value of proteins that the relation between gain in weight and protein efficiency appears not to have been carefully analyzed. In studies on the value of various supplements to white flour conducted in this laboratory, the calculation of protein efficiency apparently added no additional information over the simple measure of body weight. These studies and an additional experiment designed to study the relation of protein efficiency to gain in weight are reported in this paper.

EXPERIMENTAL

All of the studies were made with young male rats obtained from the same breeder. Their average weight at the time the various studies were started was approximately 45 gm. Groups of 5 or 6 rats were fed each ration ad libitum. The white flour was always of the same brand but 2 lots were used which contained 13.3% and 14.4% protein (N × 6.25). The protein supplements were commercial products.

All of the diets contained 12% protein (N × 6.25). In the studies on white flour, the protein was supplied by appropriate amounts of the supplement and flour. Mixtures of casein and wheat gluten supplied the protein in the other experiment. Salt mixture 4% (Hegsted et al., '41), K_2HPO_4, 0.5%, corn oil 4% [2] were always added and the ration completed with sucrose. Crystalline thiamine, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid, and choline chloride [3] were added to all rations in the amounts reported previously (Hegsted

[2] Supplied by the Corn Industries Research Foundation, New York, N. Y.

[3] Supplied by Merck and Co., Rahway, N. J.

et al., '44), and 1 drop of haliver oil containing viosterol was given twice weekly to each rat. Each experiment was continued for 3 weeks.

Food consumption records were made by weighing the amount of food given and the amount left or spilled each day. Small food cups containing the ration were placed inside of larger dishes and papers underneath the cages recovered any spilled ration.

RESULTS

Figure 1 shows the average gain in weight and protein efficiency of the group of animals which received white flour alone or white flour and varying amounts of skim milk powder,

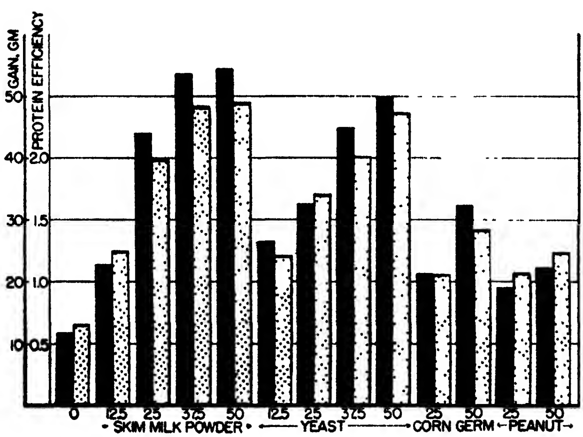


Fig. 1 Average gain in weight and protein efficiency of the groups of rats receiving white flour and white flour plus supplements. The figures on the abscissa are the per cent of the total protein supplied by the supplement. Solid bars represent gain in weight.

yeast, corn germ, and peanut flour. The differences in the supplemental value of the various proteins are apparent. Moreover, it should be noted that the proteins of the different diets are classified, relative to each other, in the same order whether protein efficiency or gain in weight is used as the measure of nutritive value.

Figure 2 is a similar chart of the data obtained using 10 diets in which the nutritive value was varied by the relative proportions of gluten and casein. This experiment was designed to give a series of proteins of gradually increasing nutritive value. It may be noted in comparing figures 1 and 2 that the protein efficiency is somewhat higher relative to the gain in weight in this experiment than in the first experiment. However, the chief point of interest is that the protein mixtures are again classified in the same relative position by either gain in weight or protein efficiency.

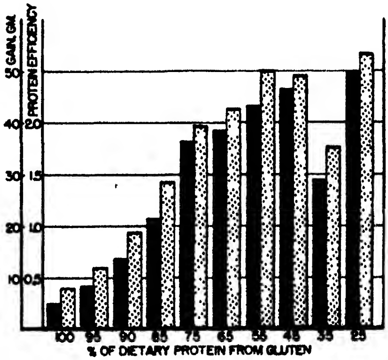


Fig. 2 Average gain in weight and protein efficiency of the groups receiving mixtures of gluten and casein. The remainder of the total protein (12% of the ration) was supplied by casein. Solid bars represent gain in weight.

This is true even for group 9 which is anomalous due to the inclusion of 2 animals which grew very poorly. One animal in this group died on the second day. The data are thus based on only 4 animals, 2 of which appeared abnormal. It seemed worthwhile, therefore, to investigate the relation between the protein efficiency and gain in weight of the individual animals.

Figure 3 is a scatter diagram showing the relation of protein efficiency to gain in weight for each of the rats in the first experiment. The straight line (no. 1) is the regression line fitted by the method of least squares. This fits the data reasonably well and the correlation coefficient is + 0.97. However, the regression line appears to fall above the experimental

points at both extremes of the data, the variation about the line increases with increases in gain in weight, and finally the line fails to pass through the origin of the graph although by definition protein efficiency is zero when the gain is zero.

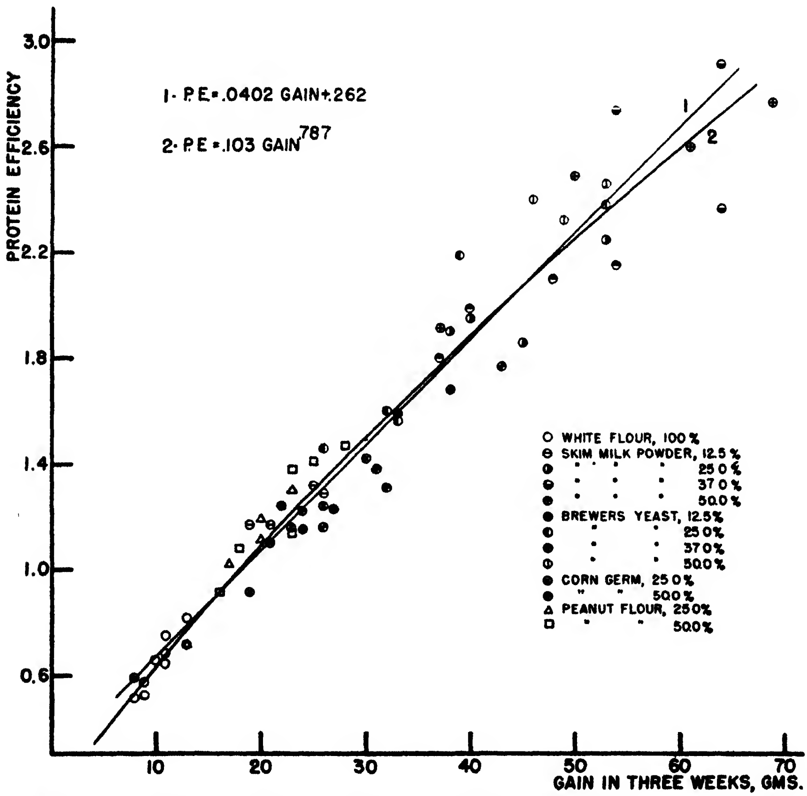


Fig. 3 The relation between the protein efficiency and gain in weight of the individual rats in Experiment I.

These difficulties disappear when the data are plotted as logarithms in figure 4.[4] The correlation is increased to + 0.984 and the data fall uniformly about the line.

The regression line log 10 P.E. = 0.787 log gain + 0.0128 may be put in terms of the original data to give the equation

[4] Protein efficiency times 10 (10 P.E.) is used rather than the original protein efficiencies for ease in plotting.

P.E. $= 0.103$ gain $^{0.787}$. The curve of this equation is shown in figure 3 as no. 2. This line fits the data at the 2 extremes better than the previous line and also approaches the origin.

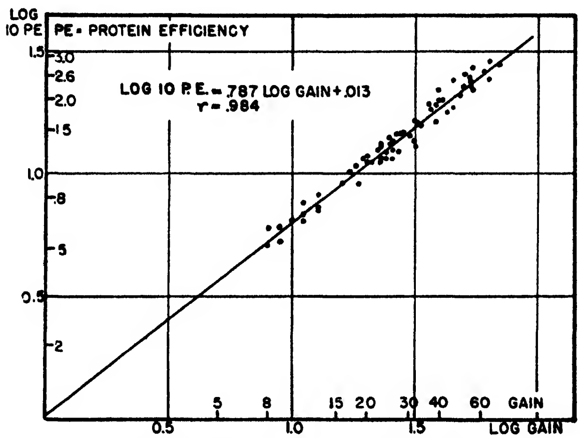


Fig. 4 The same data as in figure 3 plotted as logarithms.

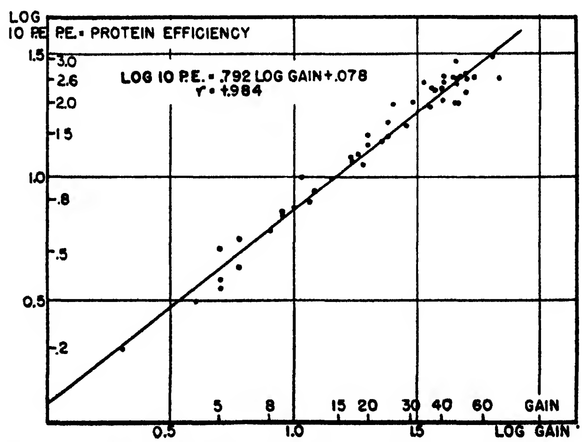


Fig. 5 A scatter diagram showing the protein efficiencies and gains in weight for the individual rats in Experiment II. The data are plotted as logarithms.

Figure 5 represents a log-log plot of the data from Experiment II. Again the data clearly fall about a straight line and the coefficient of correlation is $+ 0.984$. When this line is put in terms of the original data, the equation P.E. $= 0.0834$ gain $^{0.792}$ is obtained which is shown in figure 6.

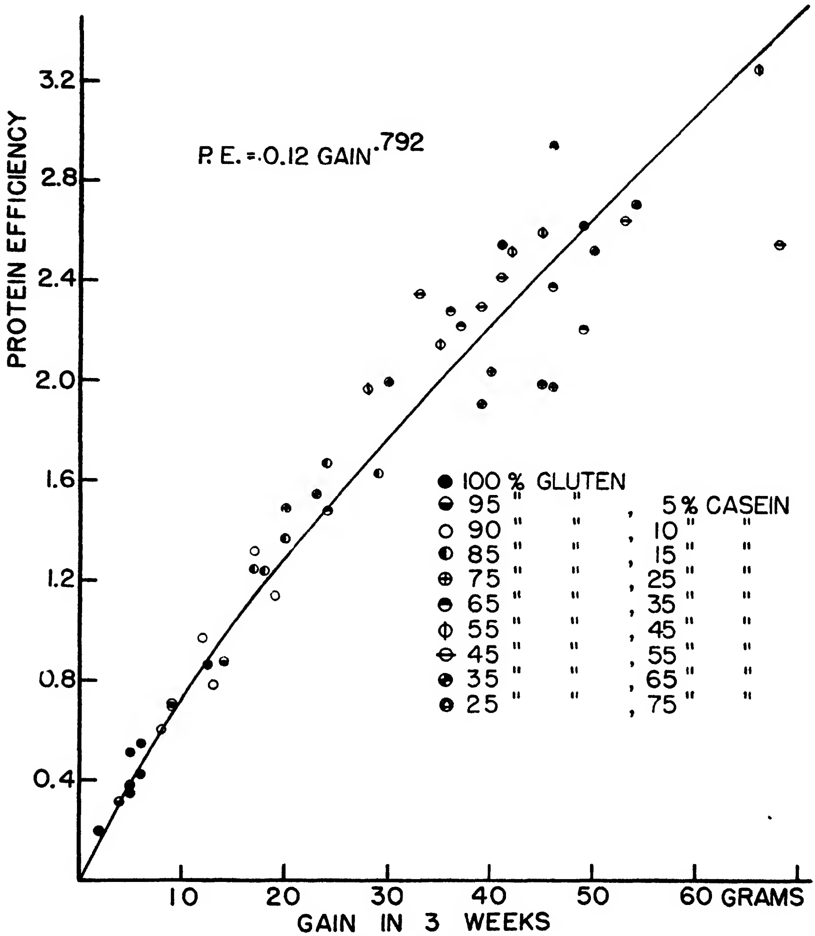


Fig. 6 The relation between protein efficiency and gain in weight in Experiment II.

DISCUSSION

In these experiments the gain in weight, amount of protein eaten and protein efficiency are known for each rat. The first 2 variables, gain in weight and the amount of protein eaten, are independent in the sense that they can be measured independently on each rat while the third variable, protein efficiency, is the ratio between the 2. The correlation between

protein efficiency and gain in weight is thus the correlation between gain in weight and a function of gain in weight. This kind of correlation has been termed "spurious" by Pearson (1897) but, as Snedecor ('46) points out, this derogatory remark would be better reserved for conclusions which have been drawn from such correlations rather than the correlations themselves.

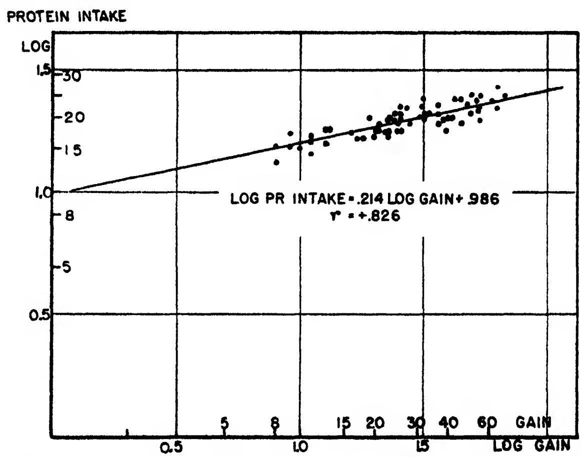


Fig. 7 A scatter diagram showing the relation of the logarithms of food intake to the logarithms of weight gains in Experiment I.

It is well known that there is in general some correlation between food intake and gain in weight. Even allowing for differences in nutritive value, animals which grow faster may be expected to have somewhat greater food intakes. In the present work, this is demonstrated in figure 7 where the logarithms of the values for protein intake in Experiment I have been plotted against the logarithms of the gains in weight. The data appear to be well represented by the regression line:

$$\text{log protein intake} = 0.214 \text{ log gain} + 0.986.$$

However, the correlation coefficient is only + 0.826 compared to the very high correlation between protein efficiency and gain in weight.

This discussion is pertinent to the present problem since the regression lines in figures 2 and 4 might be solved in the following manner:

$$\text{Log 10 P.E.} = 0.787 \text{ log gain} + 0.013 \quad (1)$$

$$\text{Since log P.E.} = \log \frac{\text{gain}}{\text{protein intake}} = \text{log gain} - \text{log protein intake}$$
$$\text{then log gain} - \text{log protein intake} + 1 = 0.787 \text{ gain} + 0.013 \quad (2)$$

$$\text{and log protein intake} = 0.213 \text{ log gain} + 0.987 \quad (3)$$

By such reasoning one thus arrives at the conclusion that the protein intake, regardless of nutritive value, is predicted by the gain in weight. However, equation 3 is the same as the equation for the regression line in figure 7 and it has already been shown by the coefficient of correlation, + 0.826, that this line predicts the protein intake only with considerable error. In fact, this coefficient of correlation indicates that only 68% of the variation has been explained by the regression line. This example thus demonstrates the erroneous conclusion which might be arrived at because of "spurious" correlation. It is clear that the excellent correlation between protein efficiency and gain in weight does not necessarily imply an equally good correlation between protein intake and gain in weight.

As has been discussed by numerous other authors, the concept of protein efficiency suffers serious theoretical difficulties. Dietary protein must be considered as fulfilling 2 functions (a) maintenance and (b) growth. Since the calculation of protein efficiency does not consider the protein required for maintenance, considerable improvement at least theoretically would result, if the maintenance protein requirement could be subtracted from the protein intake, and only the remainder considered in the calculation of protein efficiency. Boas-Fixen et al. ('34) attempted this and estimated the maintenance requirement by extending the regression line of protein intake vs. gain to the intercept of zero gain. It is readily apparent, however, that this may not be a valid procedure since undoubtedly the animals which grow have larger maintenance requirements due to the increase in body size. Furthermore, maintenance requirements cannot be estimated from the body

size since the proteins vary in nutritive value, and the maintenance requirement per unit of body size is less for the better proteins. Thus the maintenance requirement for the animals which grow (those receiving proteins of higher nutritive value) tends to be higher because of the increase in size while at the same time must be less per unit of body size than for animals which do not grow because of the difference in the nutritive value of the proteins they receive. Whether it is actually higher or lower or relatively constant for animals of varying gains will require further investigation and no satisfactory method of estimating it appears to be available at the

TABLE 1

The relative rank given the 10 proteins in Experiment II by the 3 methods of classification.

PROTEIN	AMOUNT EATEN	GAIN IN WEIGHT	PROTEIN EFFICIENCY
1	1	1	1
2	2	2	2
3	3	3	3
4	4	4	4
5	8	6	6
6	7	7	7
7	6	8	8
8	10	9	9
9	5	5	5
10	9	10	10

present time. We must, therefore, consider protein efficiency as simply a method of classifying proteins *relative to each other* as to their nutritional value. The close correlation between gain in weight and protein efficiency indicates that either of these will classify proteins as to their nutritive value in a similar manner. Since protein intake alone shows a correlation with gain in weight, it might also be used to assess the nutritive value of proteins. Table 1 shows the order in which the 10 proteins fed in Experiment II ranked according to the 3 methods. There is perfect agreement by either protein efficiency or gain in weight, but food intake alone fails to dis-

tinguish between the last 5 proteins. The same close correlation between protein efficiency and gain in weight is shown by data in the literature. As examples the data from the 2 papers by Jones and coworkers have been plotted in figure 8 as logarithms. In the first paper (Jones and Divine, '44) 16

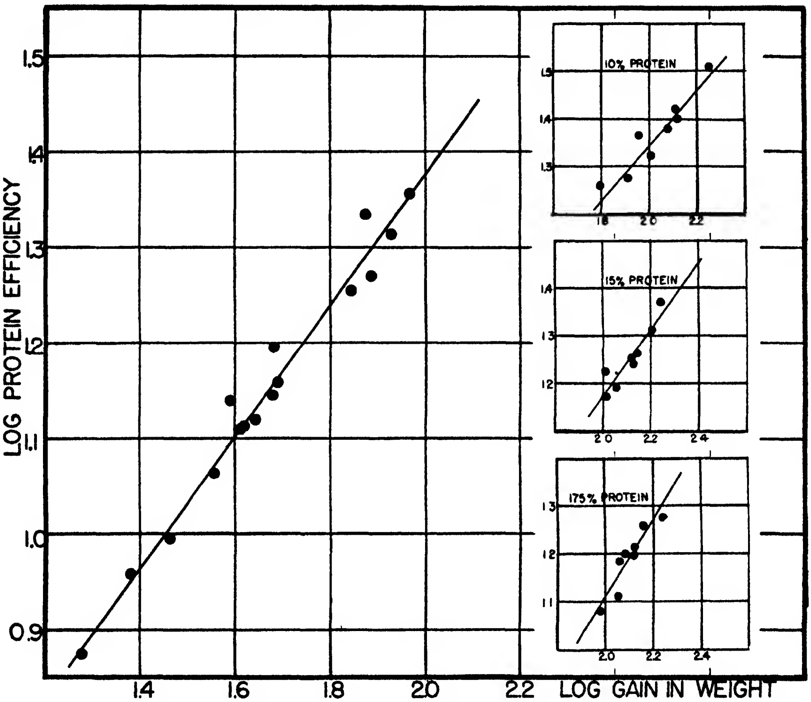


Fig. 8 The relation between protein efficiency and gain in weight in studies by Jones and Divine ('44). Insets are data from the paper by Jones and Widness ('46).

different protein mixtures were compared, all of them at a level of 9.1% protein in the diet. In the second paper (Jones and Widness, '46) 8 proteins were compared at 3 levels in the diet, namely, 10%, 15% and 17.5%. In all of these studies the correlation is very close and the points fall within the range of the standard errors of the estimated protein efficiency.

Additional examples are afforded by the recent paper of Russell et al. ('46) in which many legumes were compared as well as the effect of added methionine at 2 different levels. All of the data from this paper are shown in figure 9, and it is clear that there is a straight line relation between protein efficiency and gain in weight as in the previous data. Data obtained with chicks by Grau ('46) are shown in figure 10.

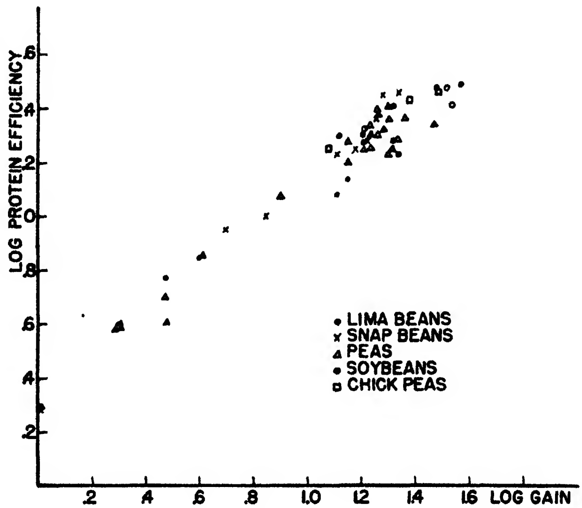


Fig. 9 The relation between protein efficiency and gain in weight in the studies by Russell et al. ('46).

In this paper 3 different proteins were studied including gluten, cottonseed and peanut proteins. Various combinations of amino acids were added to demonstrate the limiting deficiencies. A plot of these data, using food efficiency rather than protein efficiency, shows clearly that the relation is the same in chicks as in rats.

Much other data could have been selected which support the data presented in this paper. These papers were used to show that close correlation between protein efficiency and gain in weight as found under diverse conditions including many proteins, experiments involving different lengths of time, in

several laboratories, different strains of rats and even different species.

It must be stressed that this does not imply that the protein efficiency will always be the same figure for a given gain in weight. As was noted previously, the protein efficiency in figure 2 is higher relative to weight gain than in figure 1. This does not seem strange since it is well known that in repeating experiments with similar diets the absolute gain in weight may show considerable difference and protein efficiency is a function of this gain. We believe, therefore, that

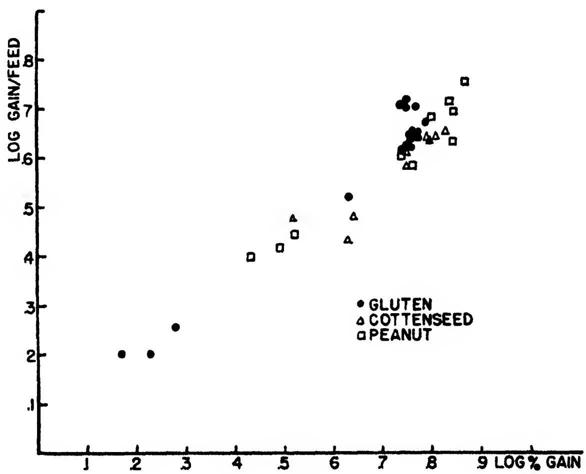


Fig. 10 The relation between food efficiency and weight gains in studies on chicks by Grau ('46).

like growth rates, protein efficiency is only relative and in the strict sense should only be used to compare the groups of animals studied at one time.

The question may be asked as to which of the 3 variables distinguishes most effectively between proteins, i.e., is 1 measure more accurate than another. In order to approach an answer to this question, variance analyses have been made of the logarithms of the 3 variables. Logarithms were used since, as previously shown, the regression lines tend to be straighter. Table 2 gives the 3 variance analyses. The 0.01

point for an F value based on 9 and 38 degrees of freedom for the greater and smaller mean square, respectively, is approximately 2.9. Consequently the variance analyses show that any 1 of the 3 variables, gain in weight, protein efficiency or amount of protein eaten, serves to bring out significant differences between the 10 proteins. The F values for gain in weight and protein efficiency are both extremely large and approximately the same size, 24.8 and 28.6. This indicates that either variable distinguishes very effectively between the proteins and it is impossible on the basis of this test to decide which is most effective.

In order to decide if gain in weight and protein efficiency can be used interchangeably in distinguishing between the proteins, the covariance between gain in weight and protein efficiency was analyzed (table 3). Comparison of the errors of estimate shows that the variances between the adjusted mean values and the observed mean values based on 9 degrees of freedom are slightly smaller than the errors of estimate determined within the protein groups. This indicates that the 2 variables, gain in weight and protein efficiency, are in fact measuring the same characteristic and that if one wishes to determine differences between proteins in terms of this characteristic, whatever it may be, one may use gain in weight alone. This is further indicated by the very high correlation

TABLE 2

Variance analyses. With respect to the 3 variables, gain in weight, protein efficiency and amount eaten.

SOURCE OF VARIATION	DEGREES OF FREEDOM	GAIN IN WEIGHT			PROTEIN EFFICIENCY			AMOUNT EATEN		
		Sum of squares	Mean squares	F	Sum of squares	Mean squares	F	Sum of squares	Mean squares	F
Total	47	6.5412			4.2360			.4119		
Between proteins	9	5.5903	.6211	24.8	3.6915	.4102	28.6	.2105	.02339	4.35
Within proteins	38	.9509	.0250		.5445	.0143		.2044	.00538	

coefficient, + 0.984, between gain in weight and protein efficiency in this experiment.

If the same sort of covariance analysis is applied to gain in weight and amount eaten the results in table 4 are obtained. The table shows that after correction for the amount eaten there is still a highly significant variance in gain in weight on the different proteins. This result is to be expected and simply means that there is a difference in the growth pro-

TABLE 3

Analysis of covariance and test of significance of adjusted protein means.[1]

SOURCE OF VARIATION	D.F.[2]	Sx^2	S xy	Sy^2	D.F	ERRORS OF ESTIMATE: y from x: S sq[3]	y from x: Mean square	x from y: S sq.[4]	x from y: Mean square
Total	47	6.5412	5.1817	4.2360	46	.1312		.2027	
Between proteins	9	5.5903	4.5358	3.6915					
Within proteins	38	.9509	.6459	.5445	37	.1058	.00286	.1847	.00499
Test of significance of adjusted means					9	.0254	.00282	.0180	.00200

[1] x = log gain, y = log protein efficiency.   [2] Degrees of freedom.

[3] $Sy^2 - \frac{(Sxy)^2}{Sx^2}$ [4] $Sx^2 - \frac{(Sxy)^2}{Sy^2}$

TABLE 4

Analysis of covariance and test of significance of adjusted protein means.

SOURCE OF VARIATION	D F	Sz^2	Sxz	Sx^2	D.F	ERRORS OF ESTIMATE: Squares[1]	Mean squares	F
Total	47	.4149	1.3608	6.4312	46	2.0781		
Between proteins	9	.2105	1.0553	5.5903				
Within proteins	38	.2044	.3055	.9509	37	.4943	.01336	
Test of significance of adjusted means					9	1.5838	.1760	13.2

[1] $x^2 = (Sxz)^2/z^2$.

moting power of proteins even though food intakes are equalized.[5]

As stated previously, the originators of this method believed that the point of maximum efficiency for each protein should be determined, and the amount of protein in the diet at these points be used in evaluating the proteins. Whether this method actually will be helpful still remains to be proven. In the final analysis the method is still one of relative classification and it is apparent that *a priori* reasoning is not sufficient grounds for stating the best method of classifying proteins. It is known already that the protein content of the diet for maximum efficiency is higher for proteins of lesser value. Thus it is not obvious that the use of the original procedure will give additional knowledge of practical value in classifying proteins over that obtained in simple feeding experiments at 1 level of protein, although this remains to be studied.

It appears to us that 1 disadvantage of the calculation of protein efficiency is that it supplies a figure for each protein which workers in the field tend to accept as characteristic of the protein. On the other hand, figures for gain in weight are always readily recognized and used in a comparative sense. Actually protein efficiency should be used in the same way and with the same reservations.

[5] The table also supplies various estimates of the correlation coefficient between gain and amount eaten. These are + 0.826 from the total, + 0.973 from the mean values, and + 0.693 from the departures within the groups. The last correlation coefficient, + 0.693, is the correlation between gain and amount eaten, independent of the type of protein fed. From this an estimate of the regression of gain in weight and amount eaten may be obtained. This regression coefficient, $\frac{Sxz}{Sx^2}$, is 1.4946 and not 1 as is assumed when the log protein efficiency, log x — log z, is computed. If this regression coefficient is a constant with various proteins and one desires to take account of the amount eaten, it should be done from this regression coefficient rather than by the simple computation of protein efficiency. Our data within groups are limited to decide this point but suggest that this regression coefficient is not a constant for the different proteins.

CONCLUSION

The nutritive value of 23 different proteins or protein mixtures was investigated by feeding experiments with 111 young rats. All diets contained 12% protein. An analysis of the data shows:

1. There is a very high correlation between gain in weight and protein efficiency (gain per gm of protein eaten).
2. Protein efficiency is a function of gain in weight rather than a characteristic of the protein fed.
3. Proteins are classified relative to each other in the same manner, and with equal accuracy, by either gain in weight alone or protein efficiency.
4. In studies on the relative nutritive value of various proteins using growing rats fed ad libitum, little additional information is gained by taking into account the amount of protein eaten, i.e., the calculation of protein efficiency.
5. The results are clearly supported by numerous data in the literature.

ACKNOWLEDGMENT

The technical assistance of Miss Alice Hay and Miss Virginia Kent in this study is gratefully acknowledged.

LITERATURE CITED

Boas-Fixen, M. A., J. C. D. Hutchinson and H. M. Jackson 1934 The biological value of proteins. V. The comparative biological values of the proteins of whole wheat, whole maize and maize gluten, measured by the growth of young rats. Biochem. J., *28:* 592.

Grau, C. R. 1946 Protein concentrates as amino acid sources for the chick: corn gluten meal, cottonseed meal, and peanut meal. J. Nutrition, *32:* 303.

Hegsted, D. M., J. M. McKibbin and F. J. Stare 1944 The effect of atabrine on choline deficiency in the young rat. J. Nutrition, *27:* 149.

Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. B. Hart 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

Jones, D. B., and J. P. Divine 1944 The protein nutritional value of soybeans, peanut, and cottonseed flours and their value as supplements to wheat flour. J. Nutrition, *28:* 41.

Jones, D. B., and K. D. Widness 1946 The comparative growth-promoting value of the proteins of wheat germ, corn germ and of some other protein foods of plant and animal origin. J. Nutrition, *31:* 675.

MITCHELL, H. H. 1924 The nutritive value of proteins. Physiol. Rev., *4:* 424.

——— 1942 Biological methods of measuring the protein values of foods. J. Animal Sci., *2:* 263.

OSBORNE, T. B., L. B. MENDEL AND E. L. FERRY 1919 A method of expressing numerically the growth-promoting value of proteins. J. Biol. Chem., *37:* 223.

PEARSON, K. 1897 Mathematical contributions to the theory of evolution: on a form of spurious correlation which may arise when indices are used in the measurement of organs. Proc. Royal Soc. of London, *60A:* 489.

RUSSELL, W. C., M. W. TAYLOR, T. G. MEHRHOF AND R. R. HIRSCH 1946 The nutritive value of the protein of varieties of legumes and the effect of methionine supplementation. J. Nutrition, *32:* 313.

SNEDECOR, G. W. 1946 Statistical Methods. The Iowa State College Press, Ames, Iowa.

REPRODUCTION AND LACTATION IN HIGHLY INBRED STRAINS OF MICE ON SYNTHETIC DIETS [1]

PAUL F. FENTON [2] AND GEORGE R. COWGILL,

WITH THE TECHNICAL ASSISTANCE OF MARIE A. STONE

Yale Nutrition Laboratory, Department of Physiological Chemistry, Yale University, New Haven, Connecticut

(Received for publication February 19, 1947)

The mouse has proved to be unusually valuable for the study of numerous problems in medicine and allied fields, notably those related to cancer and certain infectious diseases, concerning which there have been some suggestions in the literature that dietary factors may play a role. Unfortunately, this species has not been as well standardized nor have its nutritional requirements been as well elucidated as have those of the rat. Therefore it has been difficult to study the problems of cancer and infectious disease on the mouse using highly artificial diets so constructed and controlled as to have only one variable of interest. In this institution many highly inbred strains of mice with characteristics important for students of cancer have been developed by Dr. L. C. Strong. The origin of a number of these strains has been discussed elsewhere (Strong, '42).

In some preliminary dietary studies of this general problem Rogers, McElroy and Cowgill ('42) succeeded in carrying mice of the C_{57} strain (characterized by low incidence of

[1] The expenses of this study were defrayed by a grant from Nutrition Foundation, Inc., and also by grants from the American Cancer Society and The Anna Fuller Fund.

[2] Nutrition Foundation Fellow, 1945–46.

spontaneous breast cancer) to the second generation of offspring (F_2) on a purified diet containing as the component of uncertain composition a "rice polish filtrate factor II." Efforts to repeat this with other strains, however, met with failure. Foster et al. ('43) made considerable progress in this field. Cerecedo and Vinson ('44) reported that the poor lactation of mice on purified diets could be improved by the addition of a crude folic acid preparation. This work was recently repeated using crystalline folic acid (Cerecedo and Mirone, '47). The nutritional requirements of the mouse as recorded in the literature up to 1944 have been reviewed by Morris ('44).

In view of the apparent strain differences in nutritional requirements already observed by Rogers, McElroy and Cowgill ('42), it was decided to investigate this question in greater detail. We have already demonstrated [3] that the riboflavin requirement for growth is less for mice of the C_{57} strain than for the A strain (albinos with high incidence of spontaneous mammary tumors among mated females). Others have reported that it is much more difficult to maintain the A strain presumably because of either poor reproduction or poor lactation, or both. In this paper are reported favorable results obtained with respect to reproduction and lactation in the C_{57} and A strains fed highly artificial rations.

EXPERIMENTAL

This study was begun with mice of the C_{57} and A strains received from Dr. L. C. Strong and Dr. A. Gorbman.[4] Most of the animals were 21 to 28 days old at the time. One group of each strain was fed commercial stock rations [5] (in a few instances experimental stock rations); the other group received diet 101. The composition of all synthetic rations is given in table 1. A few animals were raised from weaning on diet 104. In later experiments offspring of these groups of

[3] To be published.

[4] Department of Anatomy, Yale University.

[5] Purina Dog Chow, later Purina Laboratory Chow.

TABLE 1

Composition of diets.

DIET NO.	101	104	108	111	112	114	116	119	132	133
Casein — Labco, gm	23	23	23	23	23	30	23	23	30	30
Dextrose, C.P., gm	60	57	57	60	60	45	60	60	52.5	
Dextrin, gm										52.5
Crisco, gm	10	10	10	10	10	15	10	10		
Corn oil, gm									10	10
Salts — Sure's no. 2, gm	5	5	5	5	5	7	5	5	5	5
Ruffex, gm	2	2	2	2	2	3	2	2	2	2
Cystine, gm					0.2				0.5	0.5
Rice polish filtrate factor II,[1] gm			3							
Liver powder,[2] gm		3								
Cod liver oil concentrate, gm	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.2
Linoleic acid, gm	0.27	0.27	0.27	0.27	0.27	0.41	0.27	0.27		
Choline, mg	150	150	150	150	150	196	150	150	150	150
Paba, mg	100	100	100	100	100	131	100	100	100	100
Inositol, mg	100	100	100	100	100	131	100	100	100	100
Thiamine, mg	0.5	0.5	0.5	0.5	0.5	0.65	0.5	0.5	0.5	0.5
Pyridoxine, mg	0.5	0.5	0.5	0.5	0.5	0.65	0.5	0.5	0.5	0.5
Riboflavin, mg	1	1	1	1	1	1.3	1	1	1	1
Nicotinic acid, mg	1	1	1	1	1	1.3	1	1	1	1
Calcium pantothenate, mg	3	3	3	3	3	3.9	3	3	3	3
Biotin, mg				0.016			0.015		0.02	0.02
Folic acid, mg							0.24	0.24	0.05	0.05
α-Tocopherol, mg	6	6	6	6	6	9	6	6	6	6
2-methyl-1,4-naphthoquinone, mg									1	1

mice (F_1 generation) were used. Since the belief is held in some quarters that mice will mate satisfactorily only if the male and female are raised together in the same cage, this practice was followed in the earlier experiments. From a nutritional viewpoint, if growth performance is to be measured, this system has obvious disadvantages. Consequently in most of our experiments the animals were raised from weaning in individual cages and were mated after they had reached an age of at least 70 days. The cages have wire screen bottoms and fronts, are suspended from metal shelves and are of all-metal construction. Food and water were supplied ad libitum. The diets were prepared usually at least once a week, often more frequently; between feedings they were stored in the cold. Growth rates on the stock rations and on diets 101, 104 and 114 were excellent. Growth performance on the remaining diets has not been determined.

When an animal was observed to be pregnant, it was separated from the male and placed in an individual cage supplied with nesting paper. The details regarding exact equipping of these cages were evolved gradually in the course of the early experiments. A piece of wire screen the same size as the rectangular cage bottom but of somewhat smaller mesh was turned up at the front and back to form a basket (the sides of the cage serving simultaneously as the sides of the basket). Into this the nesting paper was placed. This nest occupied the rear half of the cage. The food cup was placed in the front half of the cage, and the water bottle was attached to the front of the cage with a glass delivery tube passing through the screen to the inside. The chief advantages of this system are (a) that the nesting paper is usually kept away from the water tube and (b) that the animal quickly habituates itself to dropping the feces in the front part of the cage where they can fall through the bottom. Early experiments showed that nesting paper placed indiscriminately in the cage was invariably pushed against the water bottle outlet causing a rapid emptying. The prevention of coprophagy is a fundamental problem in nutrition and has not

been satisfactorily solved even for simple growth or maintenance experiments. The problem becomes even more acute when it is necessary to prevent the newly born mice from falling out of the cage. The method described here has, by observation, reduced coprophagy to a minimum. It is recognized, however, that the opportunities for ingestion of feces are greater than they would be in the original wire-bottom cage.

The cages containing pregnant animals were examined at least once a day. When the young were born, the female was weighed as was the entire litter. The number of offspring was reduced to 8 at birth and to 6 at the end of 1 week. The lactating females and the litters were weighed once a week and the young weaned at 21 days of age. After weaning or after the death of the last offspring in cases of lactation failure, the female was allowed to rest at least 2 weeks before being remated. Biotin assays were done by the microbiological method of Wright and Skeggs ('44) using *L. arabinosus*. This procedure was checked with an *L. casei* method. Numerous recovery experiments were carried out to test the validity of the assays, and the recoveries were entirely satisfactory. Hydrolysis of the tissue with 6 N acid was found to cause some loss of biotin, and therefore hydrolysis was done with N acid which was found to give maximal yields of biotin.

RESULTS

The data on reproduction and lactation are summarized in tables 2, 3 and 4. Mice raised on stock diets have given satisfactory breeding performance during the year. On the other hand on the purified diet (no. 101) 21 animals produced litters, 20 of which died within 3 days after birth. One litter (C_{57}) survived 14 days (table 2). Four females had been set aside as pregnant but no litters were found. It seems likely that the offspring were consumed by the mother prior to the daily inspection. No pregnancy was observed in 5 of the animals on the purified diet. Three litters were accidentally lost. When the dead young were analyzed for biotin, they

contained on the average 0.064 μg per gm of wet tissue (49 determinations on 14 animals; range 0.032 to 0.094 μg). Offspring of animals on stock rations contained on the average 0.172 μg of biotin per gm (53 determinations on 6 animals; range 0.159 to 0.184 μg). Earlier work carried out in this laboratory by A. E. H. Houk [6] and G. Keefe had shown essentially the same results. Mice of the C_{57} strain were fed rations nearly identical with our diet 101. A total of 52 litters were obtained but only 3 young were successfully weaned.

Following a 2-week rest period the females were remated. Some were continued on diet 101; others which had been raised on diet 101 were put on diets 104, 108, 111, 112, 114, 116 and 119. For subsequent matings these animals were further interchanged among the several diets. On diet 104 (liver supplement) 3 litters were weaned and the survival time of several others was increased. The addition of 16 μg of biotin (biotin concentrate) per 100 gm of diet had no beneficial effect under the conditions of this experiment. On diet 112 (cystine supplement) 2 litters, a total of 3 young were weaned (both by the same animal). Increasing the protein, fat, vitamin, salt and roughage content of the diet at the expense of carbohydrate (diet 114) was without effect. Addition of folic acid (diet 119) made it possible for 1 litter to be weaned. When both biotin and folic acid were added (diet 116), 4 litters were weaned by 2 females. Two animals were changed from the synthetic diet to a stock ration; both successfully weaned litters after this change. One of these animals was later returned to diet 101 after which the next litter died soon after birth. Young born to the animals which had been continued on diet 101 all died within 3 days after birth. Three animals raised from weaning on diet 104 were mated, and 1 litter of 5 young were successfully weaned. Five animals of our F_1 generation (from stock colony) were raised from weaning on diet 101 and placed on diet 116 at the time of mating. Of 5 litters born 1 was successfully weaned. Five other animals were raised from weaning on diet 116, but none succeeded in weaning their first

[6] Nutrition Foundation Fellow, 1943–44.

TABLE 2

Lactation performance of mice raised on synthetic diets.

DIET	FIRST GENERATION: First litter: Litters born	FIRST GENERATION: First litter: Litters weaned	FIRST GENERATION: First litter: Young weaned	FIRST GENERATION: Subsequent litters: Litters born	FIRST GENERATION: Subsequent litters: Litters weaned	FIRST GENERATION: Subsequent litters: Young weaned	F_1 GENERATION: First litter: Litters born	F_1 GENERATION: First litter: Litters weaned	F_1 GENERATION: First litter: Young weaned	F_1 GENERATION: Subsequent litters: Litters born	F_1 GENERATION: Subsequent litters: Litters weaned	F_1 GENERATION: Subsequent litters: Young weaned
101	21	0	0	4	0	0						
104				6	3	11						
108				4	1	6						
111				8	0	0						
112				6	2	3						
114				4	0	0						
116				10	4	18	10	1	2			
119				4	1	3						
L[1]				2	2	8						
132							10	4	19	8	2	9
133							6	1	6	19	12	60

[1] Purina Laboratory Chow.

TABLE 3

Lactation performance of mice raised on stock rations and placed on diet 101 at the second mating.

ANIMAL NO.	STRAIN	FIRST LITTER DIET L[1]: No weaned	FIRST LITTER DIET L[1]: Total wt.	FIRST LITTER DIET L[1]: No. dead	SECOND LITTER DIET 101: No. weaned	SECOND LITTER DIET 101: Total wt.	SECOND LITTER DIET 101: No. dead	THIRD LITTER: Diet	THIRD LITTER: No. weaned	THIRD LITTER: Total wt.	THIRD LITTER: No. dead
			gm			gm				gm	
1233	C_{57}	4	28.3	7	(young dead in 16 days)		11				
1240	C_{57}	2	14.5	5	(young lost accidentally)			101	(young dead in 2 days)		8
1249	C_{57}	2	14.6	2	6	30.3	1	101	(young dead in 3 days)		2
1250	C_{57}	6	49.2	0	6	41.8	0	101	1	4.6	1
1253	C_{57}	6	46.4	1	6	39.3	0	101	6	38.2	1
3232	A	5	30.3	0	(young dead in 5 days)		3	L[1]	5	44.3	0
3239	A	6	41.1	0	5	27.9	2	L	6	48.8	0
3249	A	6	39.4	0	(young dead in 1 day)		6	L	5	52.7	0

[1] Purina Laboratory Chow.

TABLE 4

Weight changes of females and offspring on diets 132 and 133.

	DIET 132: 6 LITTERS				DIET 133: 13 LITTERS			
Weeks after parturition	0	1	2	3	0	1	2	3
Average wt. of females, gm	26.3	27.7	29.3	27.3	27.0	27.3	26.9	26.4
Total no. of young	36	32	30	30	82	76	70	66
Average litter wt., gm	7.9	17.8	33.0	44.5	8.3	20.8	34.8	42.5
Average individual wt. of young, gm	1.3	3.3	6 6	8.8	1.3	3.6	6.5	8.4

litters. When these animals were later placed on diets 132 and 133, lactation performance was far better.

In 2 instances the young born to a female on diet 101 were exchanged with the young of a female on stock ration. Both times the stock animals successfully weaned the young, while 1 female on the synthetic diet carried 1 offspring to age 21 days when it weighed only 3.7 gm.

Three stock animals were transferred to diet 101 at parturition. All 3 litters were successfully weaned. Eight females which had been raised on stock rations and had successfully weaned 1 litter each were placed on diet 101 when they were mated a second time. The definitely adverse effects of this change on most of the animals are shown in table 3 along with results of subsequent matings.

The females of an F_1 generation group raised on synthetic diets were mated when at least 75 days old and were then placed on diets 132 and 133. The results are shown in table 2 in which are also included those F_1 animals which had been bred once when on diet 116. By employing diets 116, 132 and 133 it has been possible to carry a number of animals to the third generation entirely on synthetic diets. In table 4 are shown the weight changes of females and litters successfully raised on diets 132 and 133.

DISCUSSION

It has been shown that diet 101 (which supports excellent growth) is utterly inadequate to permit successful weaning of the offspring. The fact that offspring of females on this diet could be successfully raised if foster-nursed by females on stock diets suggests that the problem is chiefly one of lactation. The complete failure of our efforts to secure lactation on diets 101 and 114 is most interesting in view of the partial success reported by Cerecedo and Vinson ('44) with their diets R-5 and C-28, which closely resemble ours in composition. The difference in results is perhaps partly due to differences in housing the breeding colony. The partial success obtained by Foster et al. ('43) may perhaps be ascribed to

their use of vitamin K in their diet since Jukes ('40) has stressed the importance of this factor during the lactation period of the rat. Kennedy and Palmer ('45) showed biotin to be essential for reproduction and lactation of the rat.

The addition of a liver powder (diet 104) resulted in some improvement in lactation, but performance was not equal to that obtained on stock rations. The incorporation of cystine alone (diet 112) was of extremely doubtful value. Biotin concentrate was ineffective at the level employed (diet 111). Merely increasing some of the constituents of diet 101 at the expense of carbohydrate (diet 114) was equally futile. Only when folic acid or folic acid and biotin were added to diet 101 was any appreciable success obtained.

The fact that females which had been on stock rations could be transferred to diet 101 at parturition and could successfully wean their litters shows that the essential factor or factors were depleted at a rather slow rate. This was again brought out in the experiments in which stock animals were placed on diet 101 at the time of second mating. In these experiments the C_{57} strain appeared to do better than the A strain.

After the partial success of diets 116 and 119 it was decided to prepare rations which contained in addition to the ingredients of diet 101 most of the factors which have been reported to be important in lactation. The feeding of these new diets (132 and 133) did indeed result in markedly improved reproduction and lactation performance. It remains to be determined which of the dietary components may be safely omitted. Further modification of the diet can be expected to bring about further improvement in reproduction and lactation.

SUMMARY AND CONCLUSIONS

Reproduction and lactation has been studied in highly inbred strains of mice fed purified diets. A diet containing vitamins A, D and E as well as thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, choline, inositol and p-aminobenzoic acid was utterly inadequate for this purpose. The problem appears to be one of lactation. Addition of folic acid and folic

acid plus biotin gave some success. Addition of biotin, folic acid, cystine and vitamin K gave much better results.

ACKNOWLEDGMENTS

We wish to thank Dr. D. F. Robertson of Merck and Co. for furnishing us with B-complex vitamins, particularly biotin, and Dr. T. H. Jukes of Lederle Laboratories who provided us with folic acid.

LITERATURE CITED

CERECEDO, L. R., AND L. MIRONE 1947 The beneficial effect of folic acid (Lactobacillus casei Factor) on lactation in mice maintained on highly purified diets. Arch. Biochem., *12:* 154.

CERECEDO, L. R., AND L. J. VINSON 1944 Growth, reproduction, and lactation in mice on highly purified diets, and the effect of folic acid concentrates on lactation Arch. Biochem., *5:* 157.

FOSTER, C., J. H. JONES, F. DORFMAN AND R. S. KOBLER 1943 Reproduction and lactation of mice on highly purified diets. J. Nutrition, *25:* 161.

JUKES, T. H. 1940 Reproduction in rats on synthetic B-complex supplement. Proc. Soc. Exp. Biol. Med., *45:* 625.

KENNEDY, C., AND L. S. PALMER 1945 Biotin deficiency in relation to reproduction and lactation. Arch. Biochem., *7:* 9.

MORRIS, H. P. 1944 Review of the nutritive requirements of normal mice for growth, maintenance, reproduction, and lactation. J. Nat. Cancer Inst., *5:* 115.

ROGERS, L. K., L. W. MCELROY AND G. R. COWGILL 1942 Successful rearing of a second generation of mice on an artificial diet. Science, *95:* 203.

STRONG, L. C. 1942 The origin of some inbred mice. Cancer Research, *2:* 531.

WRIGHT, L. D., AND H. R. SKEGGS 1944 Determination of biotin with Lactobacillus arabinosus. Proc. Soc. Exp. Biol. Med., *56:* 95.

INDEX

Date of Issue	Date of Issue	Date of Issue
12.12.57		